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Biological Synthesis of Sulphuric Acid Esters of Steroid Hormones or their Metabolites

By R. H. De Meio, Christine Lewycka, Martha Wizerkaniuk* and Ona Salciunas
Department of Biochemistry, Jefferson Medical College, Philadelphia, Pa., U.S.A.

(*Received* 5 *October* 1956)

The synthesis *in vitro* of the sulphuric acid esters of ordinary phenols by rat-liver preparations has been demonstrated by De Meio & Tkacz (1952), De Meio, Wizerkaniuk & Fabiani (1953), De Meio, Wizerkaniuk & Schreibman (1954) and Bernstein & McGilvery (1952*a*, *b*). De Meio & Lewycka (1955) were able to observe the formation *in vitro* of dehydro*epi*androsterone sulphate by the same type of preparations and Roy (1956) has presented other experimental evidence for the formation of dehydro*epi*androsterone sulphate and other steroid sulphates. The requirements for such a synthesis appear to be the same as those found for the formation of phenyl sulphates (De Meio, Wizerkaniuk & Fabiani, 1953; Bernstein & McGilvery, 1952*a*). Direct evidence is presented in this paper for the biosynthesis of the sulphates of dehydro*epi*androsterone, testosterone, oestrone and oestradiol-17β. It is also shown that ox-liver preparations act on dehydro*epi*androsterone and oestradiol-17β, producing other metabolic changes.

EXPERIMENTAL

The techniques used for this work are the same as those described in a previous note (De Meio & Lewycka, 1955), with some additions.

Tissue preparations. The preparations from ox and rabbit livers were obtained in a similar way to those from rat liver. The microsome-free solution was obtained by high-speed centrifuging from a 20% (w/v) liver homogenate (De Meio *et al.* 1953) and the fraction precipitated between 1·2M- and 2·3M-ammonium sulphate concentrations was prepared from this solution. Microsome-free solutions or ammonium sulphate fractions from rat, ox or rabbit liver and containing 15–20 mg. of protein were added to each vessel before incubation.

Substrates. Dehydro*epi*androsterone (DHA), testosterone, oestrone and oestradiol-17β were used in amounts corresponding to 0·21 μmole and added in 0·2 ml. of 95% ethanol to a final volume of 3 ml./vessel.

Medium. The other components of the medium used for the 2 hr. incubation at 37·5° were the following: 150 μmoles of potassium phosphate buffer (pH 7), 15 μmoles of $MgCl_2$, 45 μmoles of K_2SO_4, 15 μmoles of adenosine triphosphate (disodium salt, tetrahydrate).

Determination of steroids. After deproteinization of the contents of the vessel by addition of 3 vol. of redistilled 95% ethanol, the free DHA, oestrone and oestradiol-17β were extracted with peroxide-free ethyl ether, and the bound forms of the same compounds were extracted with butanol, as described previously (De Meio & Lewycka, 1955). After evaporation of the solvent the residue was dissolved in redistilled 95% ethanol and DHA and oestrone were determined colorimetrically by the Zimmermann reaction according to the technique of Pincus & Pearlman (1941), with the Evelyn photoelectric colorimeter and filter no. 515. Oestradiol-17β was determined on the dry ether or butanol residue by the Kober method as described by Stevenson & Marrian (1947). The average blank values for the ether and butanol extracts corresponded to 6 and 11·5 μg. of DHA respectively for samples derived from incubation with microsome-free solution and to 4·2 and 8·7 μg. of DHA for those obtained with the ammonium sulphate fraction. The corresponding values for the Kober reaction were 11·8 and 15·5 μg. for the microsome-free solution and 4·5 and 4·7 μg. (expressed as oestradiol) for the ammonium sulphate fraction. Blank values were obtained for each separate determination and subtracted from the values found for the samples. The recovery of steroids and of the available steroid sulphates (DHA and oestrone sulphate) added to the samples was about 75–80%.

Preparation of extracts for paper electrophoresis. Direct proof for the formation of the sulphate was sought by incubating DHA, oestrone, oestradiol-17β and testosterone with rat- and rabbit-liver preparations, in an amount corresponding to 0·21 μmole under the conditions indicated above, but with added radioactive sulphate (0·3 μc/μmole of sulphate). After incubation, ethanol was added to each vessel to a concentration of 75% (v/v), the precipitated proteins were separated by centrifuging and the ethanol supernatant was combined with three 2 ml. ethanol washings of the precipitated proteins. The ethanolic solution was neutralized with ammonia solution to prevent hydrolysis of the sulphates, and evaporated to dryness at 30° under reduced pressure. The residue was dissolved in 3 ml. of triple-distilled water and extracted four times with 3 ml. quantities of butanol. The combined butanol extracts, neutralized with ammonia solution, were evaporated to dryness at 30° under reduced pressure; the residue was dissolved in 40–80 μl. of triple-distilled water and applied in amounts of 25–50 μl. on paper strips for subsequent electrophoresis.

Paper electrophoresis. Whatman no. 1 paper strips (11 cm. × 52 cm.) soaked in 0·05M-sodium acetate buffer, pH 4, and lightly blotted were used throughout. The 25 or 50 μl. of sample was applied as a spot to the paper prepared as indicated above and subjected to electrophoresis. The electrophoresis was carried out at 750 v and a current of 2–30 mA for 2 hr. at room temperature, with an apparatus

* Deceased November 1956.

similar to that used by Markham & Smith (1952). At the end of electrophoresis the paper strip was dried and tested for the migration of the steroid and for radioactivity.

Detection of coloured and radioactive spots. DHA, testosterone and oestrone were detected by spraying the paper with the Zimmermann reagent, according to the technique described by Bush & Willoughby (1957), and subsequent drying under an infrared lamp. The presence of the compound was indicated by the appearance of a purplish-pink spot. Testosterone was also detected by its ultraviolet absorption under a Mineralight ultraviolet lamp with maximum emission at 2537 Å. Oestradiol was detected by the technique of Barton, Evans & Gardner (1952), which gives a blue spot when the paper strip is treated with the ferric chloride–potassium ferricyanide reagent. The test is not specific, but the fact that no coloured spot was observed on material obtained by incubating the preparation without added oestradiol-17β, and that no spot appeared on spraying with the Zimmermann reagent, indicates that it was probably produced by oestradiol.

The radioactive spots were detected by scanning the paper strip with an automatic scanner consisting of a Geiger–Müller end-window counter attached to an automatic recorder.

Incubation with 'active sulphate' intermediate. The formation of DHA sulphate was tested by incubation of 0·21 μmole of DHA, an amount of transferring enzyme (obtained as described by De Meio, Wizerkaniuk & Schreibman, 1955) corresponding to 10 mg. of protein, 60 μmoles of potassium phosphate buffer, pH 7, and 'active sulphate' intermediate prepared with radioactive sulphate as described by De Meio & Wizerkaniuk (1956) in a final volume of 3 ml. for 2 hr. at 37°. The incubated material was treated as described above and subjected to paper electrophoresis, and the DHA sulphate and radioactivity were detected as already mentioned.

The ^{35}S was supplied as sulphuric acid in dilute hydrochloric acid by the Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A., on allocation from the U.S. Atomic Energy Commission. Adenosine triphosphate was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. and the reduced triphosphopyridine nucleotide from Sigma Chemical Co., St Louis, Mo., U.S.A. The ox liver was kindly supplied by the South Philadelphia Dressed Beef Co.

RESULTS

The results reported in a previous note (De Meio & Lewycka, 1955) indicated that the binding of dehydro*epi*androsterone (DHA) was an enzymic process. The binding was decreased by about 66 % in the absence of added sulphate and was decreased even more when adenosine triphosphate or Mg^{2+} ions were omitted. De Meio *et al.* (1954) have shown that active ammonium sulphate fractions can be obtained from rat-liver microsome-free solution and that two activities are present in these fractions (De Meio *et al.* 1955). The activating enzyme activity remains after heating the 1·2–2·3 M-ammonium sulphate fraction for 15 min. at 52°, and the transferring enzyme after dialysis against 0·03 M-glycylglycine for 24 hr. at 0–5° or dialysis against distilled water for 9–13 hr. followed by 4 hr. contact with 0·03 M-glycylglycine at room temperature (24°). Only the combined activating and transferring enzymes show activity, a result similar to that obtained with *p*-nitrophenol as substrate (De Meio *et al.* 1955). In a representative experiment, the activating and transferring enzymes produced the binding of 3·8 and 8·9 μg. of DHA respectively and the combined enzymes changed 24·7 μg. of the DHA to the bound form, out of a total of 60 μg. The preparations that form phenyl and *p*-nitrophenyl sulphate synthesize DHA sulphate. In the presence of DHA less *p*-nitrophenol is changed to the sulphate. A decrease of 16 % in the *p*-nitrophenyl sulphate synthesized was observed when 0·72 μmole of *p*-nitrophenol was incubated, under the conditions indicated above for DHA, in the presence of 0·21 μmole of DHA.

The microsome-free solution obtained from ox liver changed 60–70 % of the DHA to products giving a negative Zimmermann test. However, the 1·2–2·3 M-ammonium sulphate fraction obtained from this preparation did not show this effect (Table 1). These results indicate that the microsome-free solution obtained from ox liver contains enzymes, other than those involved in the formation of the sulphate, which change DHA into products not reacting in the Zimmermann test. In the rat-liver microsome-free solution this activity seems to be low or absent. The transformation of DHA into products not reacting in the Zimmermann test requires the presence of adenosine triphosphate and does not require oxygen. Adenosine triphosphate can be replaced by reduced diphosphopyridine nucleotide, but not by diphosphopyridine nucleotide.

Table 1 contains representative results of experiments on the binding of oestrone. Rat- and ox-liver preparations bind oestrone, the first doing so to the same extent as for DHA.

Oestradiol-17β is apparently bound to the same extent as oestrone by rat-liver microsome-free solution, but only about a third as much as is oestrone by the ammonium sulphate fraction. The considerable decrease observed in the absence of sulphate is indirect evidence for the formation of oestradiol sulphate (Table 2). The results contained in Table 2 indicate that part of the oestradiol is changed by ox-liver microsome-free solution to a substance that gives a positive Zimmermann reaction, which is probably oestrone (Pearlman & De Meio, 1949). On the other hand, the ammonium sulphate fraction forms much less of this substance. This may also be the explanation for the difference observed between the microsome-free solution and the ammonium sulphate fraction with rat liver. A lower total recovery of oestradiol was observed with microsome-free solution than with the ammonium sulphate fraction.

Our indirect evidence (De Meio & Lewycka, 1955) indicates that the compound formed by incubation of DHA with rat-liver preparations is DHA sulphate. Direct evidence for the formation of this and other steroid sulphates was obtained through the use of radioactive sulphate and paper electrophoresis as described above.

The material obtained from incubation of DHA, testosterone and oestrone with the liver preparation gave a coloured spot which coincided with the radioactive spot, the radioactivity varying from three to ten times the background count. The coloured spots extended 7–10 cm. from the point of application and the radioactive spots 6–11 cm. The apparent difference in size in the spots is probably due to the difference in sensitivity of the two methods employed for their detection.

Under the conditions for electrophoresis used in our experiments inorganic sulphate labelled with ^{35}S migrated off the paper. The radioactivity on the paper was not due to inorganic sulphate because material obtained by addition of radioactive sulphate after incubation of DHA with unlabelled sulphate gave a coloured spot due to DHA sulphate, but no radioactive spot.

Similar evidence for the coincidence of the coloured and radioactive spots for material obtained on incubation of oestradiol-17β with the liver preparation is presented here. Coloured spots extending 2–8 cm., and radioactive spots 1–10 cm. from the point of application were obtained. The radioactivity varied from twice to more than ten times the background count. With oestradiol a second spot migrating 18–26 cm.,

Table 1. *Metabolism and synthesis of steroid sulphates by liver preparations*

Medium: 15 μmoles of $MgCl_2$, 45 μmoles of K_2SO_4, 15 μmoles of adenosine triphosphate (ATP), 150 μmoles of potassium phosphate buffer, pH 7, 15–20 mg. of protein as enzyme preparation. Total volume, 3 ml.; incubation for 2 hr. at 37·5°. DPN, Diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Preparation	Steroid	Initial steroid (μg.)	Free steroid (μg.)	Bound steroid (μg.)	Total steroid recovery (μg.)	Conditions
Microsome-free solution	DHA	60	8·6	10·0	18·6	In air
Microsome-free solution	DHA	60	10·2	11·1	21·3	In N_2
Microsome-free solution	DHA	60	43·8	8·3	52·1	No ATP
Microsome-free solution	DHA	60	47·4	8·6	56·0	10·8 μmoles of DPN (no ATP)
Microsome-free solution	DHA	60	22·3	0	22·3	10·8 μmoles of DPNH (no ATP)
Ammonium sulphate fraction	DHA	60	26·4	24·2	50·6	—
Rat-liver microsome-free solution	Oestrone	56·2	20·7	26·7	47·4	—
Rat-liver ammonium sulphate fraction	Oestrone	56·2	30	25·9	55·9	—
Ox-liver microsome-free solution	Oestrone	56·2	36·2	9·1	45·3	—
Ox-liver ammonium sulphate fraction	Oestrone	56·2	44·8	11·3	56·1	—

Table 2. *Metabolism of oestradiol-17β by liver preparations*

Medium was as given in Table 1 with the addition of 56·6 μg. of oestradiol. Dialysis was carried out for 1½ hr. against triple-distilled water.

Preparation	Free oestradiol (μg.)	Bound oestradiol (μg.)	Observations
Rat-liver microsome-free solution	8·3	27·2	—
Rat-liver microsome-free solution (dialysed)	8·3	28·5	—
	33	1·9	No sulphate
Rat-liver ammonium sulphate fraction	31·4	10·2	—
Rat-liver ammonium sulphate fraction	40·4	5·3	No sulphate
Ox-liver microsome-free solution (dialysed)	29·2	8·2	—
	36·6	0·8	No sulphate
Ox-liver microsome-free solution (dialysed)	24·3	18·3	Determined by Kober reaction
Ox-liver microsome-free solution (dialysed)	7·8*	9·5*	Determined by Zimmermann reaction
Ox-liver ammonium sulphate fraction	31·8	10·7	Determined by Kober reaction
Ox-liver ammonium sulphate fraction	0	2·5*	Determined by Zimmermann reaction

* Probably oestrone.

but less intense than the first, was repeatedly observed.

No migrating coloured or radioactive spots were obtained when the complete preparation was incubated under one of the following conditions: at 0°; with boiled-liver preparation; in the absence of sulphate or of adenosine triphosphate.

The radioactive and coloured spots coincided for DHA sulphate prepared by incubation of DHA with 'active sulphate' intermediate and transferring enzyme as indicated above.

When 10–30 μg. of DHA, testosterone or oestrone sulphate was added to an extract of a preparation incubated without the addition of the steroid and the product subjected to paper electrophoresis, the coloured spots appeared in the same position as those found for the material obtained by incubation of the preparations with the free steroids.

DISCUSSION

The fact that a threefold increase in binding of dehydro*epi*androsterone (DHA) by liver preparations is observed on the addition of sulphate is indirect evidence for the formation of DHA sulphate. The paper-electrophoresis experiments on material obtained after incubation with radioactive sulphate furnish direct evidence for the same process. The radioactive spot must be due to bound sulphate, since inorganic sulphate under identical conditions migrates off the paper. The coincidence of the radioactive and the coloured spots (Zimmermann reagent) indicates that the sulphate and DHA migrate together. As DHA does not migrate by itself, it must be bound to the sulphate. On the other hand, apart from conjugation with sulphate, DHA appears to be unchanged, since the recovery lies within that of the method (about 80%). Further evidence for the biosynthesis of DHA sulphate is that no migration of DHA is observed if the incubation is carried out in the absence of sulphate. Similar evidence is presented for the formation of the sulphates of testosterone, oestrone and oestradiol-17β. In the last case there are indications that more than one type of sulphate is formed. Theoretically three possible types of sulphate of oestradiol-17-β can exist, but only further work can determine which of these are involved in the present experiments.

Segal (1955) has presented other evidence for the formation of oestrone sulphate by use of ^{14}C-labelled oestrone as a substrate, and Roy (1956) for the formation of sulphates of DHA and other steroids.

The requirements for the formation of DHA sulphate and the other sulphates are the same as those found for the synthesis of the sulphuric acid esters of phenols (De Meio *et al.* 1953; Bernstein & McGilvery, 1952*a*).

The combined activating and transferring enzyme preparations in the presence of SO_4^{2-} and Mg^{2+} ions and adenosine triphosphate synthesize DHA sulphate and, as we have already shown, also *p*-nitrophenyl sulphate (De Meio *et al.* 1955). In the presence of DHA, less *p*-nitrophenol is changed to the sulphate. Further, DHA is changed to the sulphate by incubation with the 'active sulphate' intermediate and transferring enzyme. These observations do not prove that the same system is involved in both processes; it may well be that the two substrates compete for the 'active sulphate' intermediate (Bernstein & McGilvery, 1952*b*) but that the transferring enzyme may be different. The latter appears to occur, as Gregory & Nose (1957) reported recently the finding of two separate fractions (sulphokinases): one produces the sulphates of steroids containing a β-hydroxyl group at C-3 and the other transfers sulphate to many phenolic compounds including the steroid phenols.

SUMMARY

1. The synthesis *in vitro* of dehydro*epi*androsterone, testosterone, oestrone and oestradiol-17β sulphates with liver preparations has been demonstrated. The system requires Mg^{2+} ions and adenosine triphosphate for activity.

2. Ox-liver microsome-free solution, but not the ammonium sulphate fraction, partially changes dehydro*epi*androsterone to a substance or substances reacting negatively to the Zimmermann test. The presence of adenosine triphosphate or of reduced diphosphopyridine nucleotide is required for the process.

3. The microsome-free solution, but not the ammonium sulphate fraction, changes oestradiol into a substance (probably oestrone) giving a positive Zimmermann test.

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Extraction of Proteins other than Myosin from the Isolated Rabbit Myofibril

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Although satisfactory methods now exist for the isolation of myofibrils in appreciable quantities, little systematic combined biochemical and morphological study has yet been made of the organization of the chemical components in these structures. There is now clear evidence that the myofibril is composed mainly of the proteins myosin, actin and tropomyosin, but most investigations (e.g. Hasselbach, 1953; Hanson & Huxley, 1953; Hodge, 1955) concerned with the selective extraction of these proteins have been qualitative in nature. Furthermore, with the notable exception of the work of Hasselbach (1953), few attempts have been made to characterize and clearly identify the proteins extracted by the procedures used. Thus although the studies of Hasselbach (1953), Hanson & Huxley (1953) and Finck, Holtzer & Marshall (1956) indicate that there is a high concentration of myosin in the A band they do not allow us to decide whether this protein is present in the I band as well. Szent-Györgyi, Mazia & Szent-Györgyi (1955) have provided evidence that protein other than myosin is to be found in the A band and draw the conclusion from their results that appreciable amounts of myosin are localized in the I band.

One approach to the problem of the localization of myosin and the other myofibrillar proteins is provided by the observation (Perry, 1953) that a fraction free of myosin could be extracted from rabbit myofibrils by solutions of low ionic strength. This fraction contained two main components, one of which was shown to be tropomyosin, but the second component could not be identified with myosin or with the F or G form of actin. The present paper is concerned with the identification of the second component as a form of inactive actin and with a further investigation of the selective extraction of proteins other than myosin from the myofibril. It forms the biochemical background to the following paper (Corsi & Perry, 1958) devoted to the study of the localization of myosin, tropomyosin and actin by phase-contrast microscopy of myofibrils extracted under varying conditions of pH and ionic strength.

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METHODS

Preparation of myofibrils. In general, myofibrils were prepared from fresh back and leg muscles and from glycerol-treated psoas muscle of the rabbit by the methods already described (Perry & Grey, 1956*a*). To prevent the occurrence of spontaneous contraction during the homogenization of glycerol-treated muscle in a medium containing 0·1 M-KCl and 0·039 M-borate buffer, pH 7·1 (0·1 M-KCl–borate), it was found necessary to store the psoas-muscle strips in 50% (v/v) aq. glycerol for 2–3 weeks at −10° to deplete the endogenous adenosine triphosphate (ATP). When fresh rabbit muscle was homogenized in 0·1 M-KCl–borate, marked contraction of the myofibrils with reversal of striation usually occurred. This spontaneous contraction was reduced, but not eliminated, by lowering the KCl concentration of this medium to 0·025 M (0·025 M-KCl–borate) and carefully maintaining the temperature at 0°.

Satisfactory preparations of myofibrils, in which the I and A bands were clearly visible and in which the average sarcomere length was 2·2 μ, were obtained by carrying out the initial homogenization and the first resuspension of the minced muscle (Perry & Grey, 1956*a*) in 0·1 M-KCl containing 5 mM-ethylenediaminetetra-acetate (EDTA), pH 7. This concentration of EDTA induces relaxation in glycerol-treated fibres (Boxler, 1954; Watanabe, 1955) and strongly inhibits the magnesium-activated adenosine triphosphatase (ATPase) activity of myofibrils (Perry & Grey, 1956*a*, *b*). Subsequent washings and recentrifugings were carried out in 0·1 M-KCl–borate, which procedure removed the EDTA as judged by the behaviour of the magnesium-activated ATPase of the myofibrils to fresh applications of EDTA (Perry & Grey, 1956*b*). There was a tendency for fibrous aggregates to form during preparation by this method but they could be removed by additional short centrifugings of the dilute myofibril suspension at low-speed.

Preparation of proteins. L-Myosin was prepared by a method previously described (Perry, 1955) and actin by the method of Guba & Szent-Györgyi (1945). 'Natural' actomyosin was obtained by the extraction of myofibrils with Weber's solution (0·6 M-KCl, 0·01 M-Na_2CO_3, 0·04 M-$NaHCO_3$). The extract was centrifuged for 20 min. at 10 000 *g* and actomyosin precipitated from the supernatant by dilution with 14 vol. of water at pH 7.

Tropomyosin was prepared as described by Bailey (1948), further purified by ethanol precipitation as suggested by Bailey (1951) and recrystallized twice. This preparation, which was used to obtain the standard curve for the viscometric assay of tropomyosin, was homogeneous after electrophoresis for 7 hr. in 0·5 M-KCl containing 0·05 M-phosphate buffer, pH 6·8. Likewise only one component was apparent after centrifuging for 6 hr. at 259 000 *g* in the Spinco Analytical Ultracentrifuge under the same ionic conditions.

Extraction of myofibrillar proteins. Immediately after the myofibrils were prepared, a sample of the concentrated suspension was centrifuged for 30 min. at 20 000 *g* to estimate the soluble protein in the clear supernatant obtained (usually less than 0·5% of total N of the suspension). The concentrated preparation (3–5 mg. of N/ml.) was diluted to 0·7–0·8 mg. of N/ml. with the solution to be used as extractant and dialysed against 40–50 vol. of the extractant for 24 hr. The diffusate was replaced with a similar volume of fresh extractant and 24 hr. later the myofibrillar suspension was transferred to a stoppered flask containing a trace of toluene and minimum air space. After the required interval the suspension was centrifuged for 1 hr. at 105 000 *g* on the Spinco Preparative Ultracentrifuge. The soluble fraction had essentially the same composition if the suspension was centrifuged for 15 min. at 20 000 *g*, but the higher speed was chosen for routine analysis so that the residue sedimented to a small pellet, which was less than one-eighth of the total volume of the original suspension. Under these conditions it was assumed that the percentage of protein extracted was 100 × concentration of N in the supernatant divided by the concentration of N in the whole suspension. Storage and all manipulations were carried out at 0°.

Estimation of tropomyosin. This was carried out by dialysing the protein solution against 0·078 M-borate buffer, pH 7·1, and determining with an Ostwald viscometer at 0° the drop in viscosity on addition of sufficient 2 M-KCl (in 0·078 M-borate) to obtain a final concentration of 0·5 M (Perry, 1953). The tropomyosin concentration of the solution was determined by reference to a standard curve obtained with a highly purified sample of tropomyosin treated under identical conditions over the range 0·015–0·17 mg. of N/ml.

After dialysis for 7 days against 5 mM-acetate buffer, pH 4·3, the viscosity drop obtained under the standard-assay conditions was identical with that obtained with a control sample stored for the same period in 0·078 M-borate, pH 7·1. Dialysis against 5 mM-2-amino-2-hydroxymethyl-propane-1:3-diol (tris), pH 7·9, for 7 days, however, reduced the drop in relative viscosity from 0·79 to 0·70 in a tropomyosin sample containing 0·081 mg. of N/ml. For this reason tropomyosin estimations on samples obtained by prolonged periods of alkaline extraction of the myofibril tended to be somewhat low, and in these cases estimations were usually restricted to extracts less than 2 or 3 days old.

Electrophoresis. Most studies were carried out in 0·25 M-KCl containing 0·05 M-phosphate buffer, pH 6·6–6·8, with the Perkin–Elmer electrophoresis apparatus, model 38. At this ionic strength satisfactory separation of components without appreciable precipitation of protein was best obtained at pH 6·8. Protein solutions, concentrated when necessary by suspending them at 0° in a dialysis bag in front of an electric fan, were dialysed against the buffer for 1–2 days at 0°. For estimation of mobility the conductivity of the buffer used for dialysis was taken rather than that of the protein solution.

Sedimentation and diffusion constants. The sedimentation rate was determined in the Spinco Analytical Ultracentrifuge, model E. The Tiselius electrophoresis cell (Perkin–Elmer) was used to determine the diffusion constant. The diffusion curves were analysed by the method of Rarkham (unpublished work).

Total N determinations were carried out by the micro-Kjeldahl technique, and the method of Krebs & Hems (1953) was used for the chromatography of adenine nucleotides.

RESULTS

It became apparent in the earlier stages of this investigation that on prolonged treatment of myofibrils with 0·078 M-borate buffer, pH 7·1, about 25% of the myofibrillar protein passed into solution. This value was much higher than that obtained previously (Perry, 1953) and it was considered to represent the upper amount of protein which could be extracted under these conditions. An increase in the ionic strength of the borate buffer by addition of salts such as KCl considerably reduced both the amount of protein passing into solution and the marked swelling which is characteristic of myofibrils suspended in media of low ionic strength.

Whereas at pH 7·1 in the borate medium 15–20 days or longer were required for maximum extraction, with 5 mM-tris buffer, pH 7·7 or 8·3, 30–35% of the total myofibrillar protein passed into solution within 4–5 days. After such a period at the higher pH the myofibrillar structure usually became unrecognizable and extraction under these conditions

was of limited value for morphological studies (see Corsi & Perry, 1958). Even if extraction was prolonged usually little more than 35–36% of the myofibrillar protein dissolved in the buffer. In one experiment, however, after extraction for 59 days with 5 mM-tris buffer, pH 7·7, 42% of the total myofibrillar protein passed into solution.

Electrophoresis of the protein solution obtained by extraction of myofibrils isolated from fresh muscle with low-ionic-strength buffers (hereafter called the 'soluble fraction') showed that three main components were present (Fig. 1). These will be referred to as the *A* (tropomyosin), *B* and *C* components, in order of decreasing mobility (see Table 1). Similar results were obtained with myofibrils prepared in the 0·025 M-KCl–borate and the 0·1 M-KCl–borate–EDTA media (Fig. 1*a*, *b*).

Usually electrophoresis was carried out in 0·25 M-KCl containing 0·05 M-phosphate buffer, pH 6·8, under which conditions satisfactory separation of the components was obtained without precipitation of protein. Component *B* was the most abundant and after exhaustive extraction at higher pH values the amount present was about twice that of *A*, whereas *C* was somewhat variable in amount; with myofibrils isolated from fresh muscle, by inspection of the Tiselius diagrams it was roughly estimated to represent about 5% of the whole soluble fraction. As a rule component *A* appeared as a symmetrical peak, whereas the *B* peak sometimes showed asymmetry, particularly in the ascending limb, and on occasions after prolonged electrophoresis it appeared to be splitting into two components.

Although only slight differences in the relative proportions of tropomyosin and component *B* were noted during the progress of extraction, more marked effects were observed with myofibrils prepared from fibres which had been stored for several weeks in 50% (v/v) glycerol. Under otherwise comparable conditions the total protein extracted by 0·078 M-borate was less than that obtained from fresh myofibrils (Table 2). This was reflected in the reduced amount of component *B* and the almost complete disappearance of *C*. The extraction of tropomyosin was relatively much less

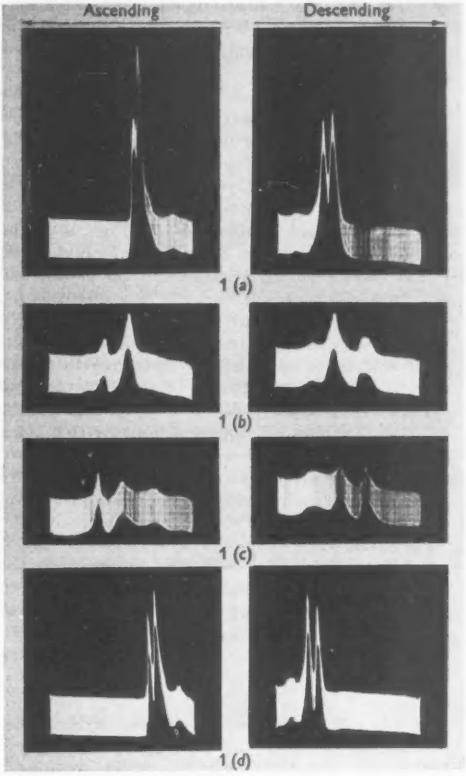

Fig. 1. Electrophoresis diagrams of solutions obtained by extraction of rabbit myofibrils with buffers of low ionic strength. Electrophoresis conditions: 0·25 M-KCl, 0·05 M-potassium phosphate buffer. (*a*) From fresh muscle extracted 9 days with 0·078 M-borate buffer, pH 7·1. 300 min. at pH 6·8. (*b*) From fresh muscle (EDTA preparation) extracted 12 days with 5 mM-tris buffer, pH 7·75. 510 min. at pH 6·8. (*c*) From fresh muscle extracted 45 days with 5 mM-phosphate buffer, pH 6·45. 480 min. at pH 6·8. (*d*) From muscle in rigor mortis extracted for 3 days with 0·078 M-borate, pH 7·1. 240 min. at pH 6·7.

Table 1. *Mobilities of components* A, B *and* C

Conditions: 0·25 M-KCl, 0·05 M-sodium phosphate buffer, pH 6·8. Mobility is expressed as 10^{-5} cm.^{2}/v/sec. Desc. and asc.: descending and ascending boundaries.

	Mobility					
	Component *A*		Component *B*		Component *C*	
Extractant	Desc.	Asc.	Desc.	Asc.	Desc.	Asc.
0·078 M-Borate, pH 7·1	3·8	3·9	3·1	3·5	1·3	1·2
0·078 M-Borate, pH 7·1 (duplicate)	4·0	4·1	3·0	3·4	1·4	1·3
5 mM-Phosphate, pH 6·45	4·0	4·2	2·8	3·2	1·4	1·6

reduced and the soluble fraction obtained from glycerol-treated myofibrils usually contained this protein as the main component (Table 2, e.g. preparation no. 164G).

As might be expected, extraction at pH 6·45 (5 or 6·7 mM-phosphate buffer) reduced the amount of protein which passed into solution. For example, after 10–15 days 8–10 % of protein was extracted and electrophoretic analysis indicated that this was due mainly to the suppression of the solubility of tropomyosin and component *B* (Fig. 1*c*). The extraction of these two components at higher pH values was considerably reduced by the presence of 0·5 mM-$MgCl_2$ (Table 2).

In the previous investigation (Perry, 1953) myosin was shown to be absent from the borate-soluble fraction and likewise under conditions of more complete extraction, i.e. with buffers of pH 7·7 and 8·3, no ATPase activity passed into solution (Table 3). It appeared unlikely that components *A*, *B* and *C* were derived by breakdown of the myosin molecule for no significant amount of protein passed into solution on treatment of L-myosin with borate. This fact, together with the finding that there was no fall in ATPase activity of myofibrils during the extraction process (on the contrary there was usually a 10–15 % increase in activity), suggested that the myosin molecule was not being degraded during the extraction.

Component A (*tropomyosin*)

It was established that component *A* is electrophoretically identical with tropomyosin (Perry, 1953). In this earlier study rabbit myofibrils isolated in 0·078 M-borate buffer contained about 4 % of tropomyosin, a result which agrees well with the figure given by Bailey (1948) for the tropomyosin which could be extracted from whole adult rabbit muscle. Our present studies indicate that at least 10–12 % of the total N of myofibrils, freed from other cell components by isolation in media of ionic strength ranging from 0·025 to 0·10, consists of tropomyosin (Table 2). Analysis of the composition of the soluble fraction extracted by 0·078 M-borate after varying intervals of time showed that the soluble fraction was most rapidly extracted during the first and second days of exposure to the borate solution (Fig. 2). Consequently, in the earlier study (Perry, 1953), considerable loss of the soluble fraction occurred during the washing by centrifuging and resuspension. These losses have now been avoided by dialysing the myofibril suspension against the extractant (see Methods). Somewhat similar values to those obtained for mixed rabbit-skeletal muscle were obtained for the tropomyosin content of myofibrils isolated from hen-breast and rat-leg muscles (Table 2).

Table 2. *Total nitrogen and tropomyosin extracted by buffers of low ionic strength*

Nitrogen soluble in the fresh myofibril preparations (usually <0·5 %) is included in the total extracted. Preparations 164G and 167G were glycerol-treated (see text). All preparations were from rabbits except 172F (hen) and 175F (rat). For definition of 'soluble fraction', see text.

Preparation no.	Extraction medium		Extraction time (days)	Total N extracted (% total N)	Tropomyosin N (% total N)	Tropomyosin N (% of N in soluble fraction)
	Preparation medium	Composition	pH			
150F	0·025 M-KCl–borate	0·078 M-Borate	7·1	40	24·5	8·6	35
151F	0·025 M-KCl–borate	0·078 M-Borate	7·1	130	21·7	—	—
164G	0·1 M-KCl–borate	0·078 M-Borate	7·1	17	17·5	11	63
167G	0·1 M-KCl–borate	0·078 M-Borate	7·1	19	15·5	7·6	49
163FV	0·1 M-KCl–borate + EDTA	5 mM-Tris	7·75	2	25·5	9·6	38
		5 mM-Tris	7·75	65	35·2	—	—
166FV	0·1 M-KCl–borate + EDTA	5 mM-Tris	8·3	3	30·5	11·5	37
		5 mM-Tris	8·3	7	32·8	(11·2)	—
172F	0·025 M-KCl–borate	5 mM-Tris	7·9	4	28·2	10·0	35
175F	0·025 M-KCl–borate	5 mM-Tris	7·8	3	21·4	7·5	35
174FV	0·1 M-KCl–borate + EDTA	5 mM-Tris + 0·5 mM-$MgCl_2$	7·9	2	9·2	4·1	36
				10	10·2	—	—
157F	0·025 M-KCl–borate	6·7 mM-Phosphate	6·45	10	8·4	2	—
160F	0·025 M-KCl–borate	5 mM-Phosphate	6·45	45	12·4	3·2	26
168FV	0·1 M-KCl–borate + EDTA	5 mM-Phosphate	6·45	17	11·6	3·3	28

Table 3. *Protein nitrogen extracted from rabbit myofibrils by buffers of low ionic strength*

Q_p = μl. of P/hr/mg. of protein.

Preparation no.	Extraction medium	Extraction time (days)	Q_p of whole suspension	ATPase soluble (%)	Protein N extracted (as % of total N)
157F	0·078 M-Borate, pH 7·1	3	792	—	—
	0·078 M-Borate, pH 7·1	23	904	Nil	26·3
160F	0·1 M-KCl–borate, pH 7·1	Freshly prepared	690	—	—
	5 mM-Tris, pH 7·8	2	824	Nil	34
163FV	5 mM-Tris, pH 7·75	76	1187	Nil	35

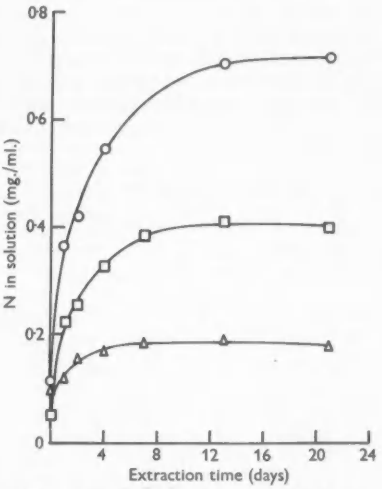

Fig. 2. Time course of extraction of proteins from rabbit myofibrils. Myofibrils were prepared in 0·025 M-KCl–borate buffer and were extracted with 0·078 M-borate at 0°. ○, Total N/ml. of soluble fraction; □, N/ml., precipitated by 0·75 M-phosphate buffer, pH 7·2; △, tropomyosin N/ml., estimated viscometrically.

Component B

On exhaustive extraction component *B* was obtained in amounts representing 15–20 % of the total myofibrillar protein and, although on quantitative grounds it seemed that this protein was derived from the myofibrillar actin, component *B* was not identical with F or G actin. Component *B* possessed pseudoglobulin properties, in that it was precipitated by 50 % saturated $(NH_4)_2SO_4$ but not by dialysis against distilled water or very low ionic-strength buffers, pH 7. It seemed likely that it was some form of inactive actin but attempts to restore its ability to undergo the G–F polymerization by treatment with ATP, ascorbic acid and $MgCl_2$ were unsuccessful. Study of component *B* was greatly facilitated by the observation that it was selectively precipitated by phosphate buffer, pH 7·2, at concentrations higher than 0·6 M. After precipitation and re-solution 90–95 % of component *B* was re-precipitated by 0·75 M-phosphate from solutions containing from 0·04 to 0·19 mg. of N/ml. It was noted that on precipitation component *B* tended to undergo irreversible changes which rendered it partly insoluble. If precipitation was carried out at 0° and the precipitate redissolved within 5–10 min. clear solutions were readily obtained. Re-solution of the precipitate was facilitated by the addition of 5 mM-ascorbic acid to the original solution of the soluble fraction. Under these conditions precipitates of component *B* could be left for longer periods without becoming insoluble. In general this protein was unstable when redissolved after precipitation. The original soluble fraction could be preserved for weeks without apparent change but solutions of precipitated component *B* progressively became turbid on standing at pH 7 and ionic strength 0·2–0·4.

F and G actin, myosin and tropomyosin were not precipitated by 0·75 M-phosphate buffer, pH 7·2, but actin irreversibly depolymerized by treatment with M-KI was precipitated. Nevertheless such depolymerized F actin did not have the same mobility as component *B* when added to a preparation of the borate-soluble fraction (Fig. 3*c*, *d*).

When F actin solutions were dialysed against 0·078 M-borate buffer, pH 7·1, for several days a steady increase in the amount of protein which could be precipitated with 0·75 M-phosphate was obtained. The properties of this protein appeared identical with those of component *B* and when added to the borate-soluble fraction it had the same mobility as component *B* and was indistinguishable from it (Fig. 3*a*, *b*).

When 'natural' actomyosin was dialysed for 9 days against 0·078 M-borate buffer, pH 7·1, a soluble fraction was obtained representing about 11 % of the total protein and 70–80 % of which could be precipitated by 0·75 M-phosphate.

These results indicated that component *B* was a form of irreversibly inactivated actin, and the low relative viscosity of its solutions suggested that it was in the depolymerized form. After phosphate precipitation the particle weight was high because,

in a medium consisting of 0·1 M-glycine, 0·1 M-KCl and sufficient N-HCl to bring the pH to 1·95, component *B* sedimented as one slightly asymmetric peak, $S_{20} \sim 16$ (one determination). A single determination of the diffusion constant in the same medium gave a value of $D_{20} = 1 \cdot 1$.

Component *B* was produced by the treatment with borate and was not originally present in the myofibril, for protein was not precipitated by 0·75 M-phosphate buffer from extracts of freshly prepared whole myofibrils made with Weber's solution and 1·65 M-LiCl.

The maximum amount of component *B* (actin) was extracted by tris buffer of low ionic strength at higher pH values (7·7–8·3) and usually amounted to 20 % of the myofibrillar protein. This would represent about 80 % of the total actin presumed to be present if the figure of 15 % (Hasselbach & Schneider, 1951) is taken for the actin content of whole muscle and the myofibrillar protein is assumed to account for 60 % of the total muscle protein.

It was noted in the studies with 0·078 M-borate, pH 7·1, that after extraction of 20–25 % of the myofibrillar protein little additional protein passed into solution even if extraction was continued for several months. Some increase in the amount extracted was achieved with the more alkaline tris buffers, but also under these conditions some of the actin resisted extraction. Myofibrils from which 35 % of the protein had been extracted would still contract on the addition of ATP although the process was slow, and if any organized structure was apparent before contraction certainly none could be recognized after it had occurred. Also extracts of these myofibrils made with Weber's solution showed a significant decrease in relative viscosity on addition of ATP under the conditions of the actomyosin test.

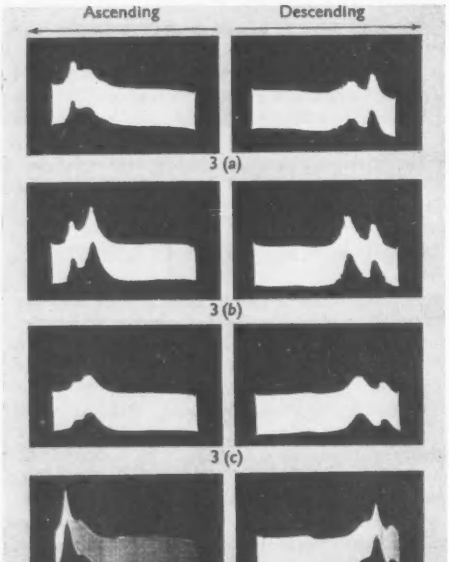

Fig. 3. Electrophoresis diagrams of 0·078 M-borate extracts of rabbit myofibrils with and without added actin preparations. Electrophoresis conditions: 0·25 M-KCl, 0·05 M-potassium phosphate buffer. (*a*) 7-day extract. 660 min. at pH 6·8. (*b*) Extract as used in (*a*) plus inactive actin obtained by prolonged dialysis of F actin against 0·078 M-borate buffer followed by phosphate precipitation. 660 min. at pH 6·8. (*c*) Ten-day extract. 720 min. at pH 6·7. (*d*) Extract as used in (*c*) plus actin treated with 1·2 M-KI. 720 min. at pH 6·7.

Component C

Component *C* was usually observed as a small peak representing about 2–3 % of the whole myofibrillar protein when extracts of myofibrils prepared from fresh muscle were made in borate and tris buffers, pH 7·1–8·3. It was also present in extracts made from myofibrils prepared from muscle allowed to go into rigor mortis (Fig. 1*d*). The amounts of component *C* in the soluble fraction were reduced if the myofibrils were washed in larger volumes of buffer than were used normally during the preparation. This component was not usually present in 0·078 M-borate extracts of myofibrils made from glycerol-treated fibres which had been stored from 2 to 3 weeks at −10°. Component *C* was apparently readily extracted at pH 6·45 from myofibrils prepared from fresh muscle, for the amounts obtained under these conditions represented about the same percentage of the total myofibrillar protein as was extracted by borate and tris buffers under more alkaline conditions (Fig. 1 and Table 2).

Extraction of soluble components together with myosin fraction

While this investigation was in progress Szent-Györgyi *et al.* (1955) reported that when the myofibril is extracted with solutions of the Guba & Straub (1943) type fortified with low concentrations of ATP (which could be expected to dissolve myosin and little actin) myosin passes into solution together with another fraction which is soluble in a medium containing 0·04 M-KCl and 0·0067 M-phosphate buffer, pH 7·2. These workers conclude that this soluble protein represents about 20 % of the myofibril and suggest that in part it is responsible for the increased optical density of the A band.

With the aim of relating these findings to our own observations we have extracted fresh and glycerol-treated myofibrils in a similar manner to that employed by Szent-Györgyi *et al.* (1955). With fresh myofibrils the non-dialysable N of the fraction remaining in solution after precipitation of myosin from these extracts invariably represented 6–7 % of the total myofibrillar protein; with glycerol-treated myofibrils it amounted to 3–4 % only. These experiments were carried out on myofibrils separated from other cell components and debris and well-washed by repeated centrifugings. It is worth noting that in our experience (with one exception of doubtful significance) it was never possible (15 experiments) to extract myosin entirely free of actin from fresh or glycerol-treated myofibrils. Extractions were carried out with varying concentrations of ATP and pyrophosphate and with added $MgCl_2$. ATP was shown to be present throughout the extraction by chromatographic methods. In these experiments the N extracted ranged from 40 to 70 % of the whole.

DISCUSSION

Extraction of myofibrils with solutions of low ionic strength, pH 6·5–8·3, brings into solution an inactive form of actin, tropomyosin and small amounts of an unidentified fraction provisionally called component *C*. Considerable swelling occurs at the higher pH values and apparently aids the extraction, which appears to be maximal when about 35 % of the myofibrillar protein is in solution. It is probable that the total myofibrillar content of these three components is about 40 %, and the fact that the myofibril disintegrates under exhaustive extraction suggests that the components of the soluble fraction have an important function in maintaining the continuity of the structural framework. It is of interest to note that even when all the myosin is extracted, i.e. in extracts containing 60–70 % of the total protein, the myofibril structure still shows organization despite the fact that the cross-band structure has been radically changed (Corsi & Perry, 1958).

Tropomyosin has been shown to be present in skeletal-muscle myofibrils in greater amounts than was originally considered to be the case. It is rather striking that tropomyosin and actin are invariably extracted together and in a fairly constant ratio (1:1·5–2·0) irrespective of the total amount of soluble fraction obtained from myofibrils prepared from fresh muscle. This observation could indicate a close association of the two proteins *in situ*.

The data presented indicate that extraction of actin by suspensions of myofibrils in media of low ionic strength in some way changes the protein so that it forms solutions of low viscosity and will no longer combine with myosin to give actomyosin which can be recognized by the conventional tests.

It must be concluded that, although part of the actin is comparatively easily extracted, about 20 % resists extraction even under more alkaline conditions. This suggests that there exists along the myofibril considerable variation in the strength of association of actin either with itself or with other protein. This observation was made on myofibrils isolated from fresh muscle and is presumably of significance in the role of this protein in myofibrillar function. The decreased solubility of the actin which occurs after prolonged storage of muscle in 50 % (v/v) aqueous glycerol is probably due to denaturation changes.

Our investigations support the view that actin, tropomyosin and myosin each exist as distinct entities in the myofibril. We have no evidence to indicate that during extraction myosin is being degraded into actin and tropomyosin, which proteins Laki (see Morales, Botts, Blum & Hill, 1955) has postulated as subunits of the myosin molecule. In fact our investigations suggest the contrary and, if taken with the findings described in the next paper, clearly indicate, as has the work of previous investigators (Hasselbach, 1953; Hanson & Huxley, 1953), that these proteins have precise localization which is dependent on the physiological state.

Component *C* is the protein of the soluble fraction which was not consistently detected and on repeated washing in 0·1 M-potassium chloride–borate the amount of this protein which could be extracted from the myofibril fell. Further, the low mobility of this fraction is reminiscent of that of the group I proteins (Jacob, 1947), which are present in extracts of whole rabbit muscle made with a solution of ionic strength 0·15 and pH 7·7. Such salt solutions do not bring into solution the myofibrillar proteins but are presumed to extract the sarcoplasm, defined as the complex system of proteins in solution which fills up the spaces between myofibrils and other formed elements within the cell. The facts suggest that component *C* arises from the sarcoplasm or possibly represents a sarcoplasmic fraction closely associated with the myofibril which is not readily removed by repeated washing. It may be dangerous to assume, however, that a component removed by prolonged washing is necessarily a contaminant of the myofibrils, particularly as one of us (S. V. Perry, unpublished work) has observed that the increased washing necessary to reduce the content of component *C* can also reduce the yield of tropomyosin extracted from the myofibril. Further investigations are in progress on the nature and origin of component *C*.

We have been unable to confirm that a protein soluble in a medium containing 0·04 M-potassium chloride and 0·0067 M-phosphate buffer, pH 7·2,

which represents 20% of the total myofibrillar protein, is extracted from the myofibril together with myosin by Guba–Straub solution containing ATP. In our hands this fraction represented 3–7% of the whole and it might be supposed that it consists in part of tropomyosin, which in all makes up at least 10–12% of the myofibril. de Villafranca (1956) has further studied this fraction, and although the effect of salt on its viscosity is remarkably like the behaviour of tropomyosin the protein is precipitated at lower ammonium sulphate concentrations; he concludes therefore that it is probably not tropomyosin. If that is so then the protein studied by Villafranca must constitute part of the residue, representing 60–65% of the myofibrillar protein left after extraction of the soluble fraction. This seems rather unlikely, as the results of the analysis of whole muscle (Hasselbach & Schneider, 1951) suggest that this residue must consist almost entirely of myosin.

SUMMARY

1. On exhaustive extraction of rabbit myofibrils with buffers of low ionic strength, pH 7·1–8·3, 25–36% of the total myofibrillar protein passed into solution. This extract contained tropomyosin, an inactive form of actin and an unidentified protein provisionally named component *C*.

2. Tropomyosin has been shown to account for at least 10–12% of the total myofibrillar protein.

3. The inactive form of actin extracted from myofibrils was electrophoretically identical with F actin inactivated by dialysis against 0·078M-borate buffer, pH 7·1, but not with F actin inactivated by treatment with potassium iodide.

4. When the extract obtained by treating myofibril with solutions which are considered to remove the A band selectively was dialysed against 0·04M-potassium chloride containing 6·7 mM-phosphate, pH 7·2, 3–7% of the total myofibrillar nitrogen remained in solution.

We wish to express our thanks to Mr T. C. Grey for skilled technical assistance and to the Medical Research Council for a research expenses grant (to S.V.P.). We are also indebted to Mr B. R. Slater for help in the determination of the diffusion constant of component *B*.

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Some Observations on the Localization of Myosin, Actin and Tropomyosin in the Rabbit Myofibril

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In the previous paper (Perry & Corsi, 1958) it has been shown that at pH 7–8, under conditions of low ionic strength, about 35% of the total protein can be extracted from the isolated myofibril. As myosin was absent from this extract, which consisted mainly of actin and tropomyosin, it seemed likely that a study of the morphological changes invölved during the extraction of myofibrils under these conditions would provide useful information on the localization of actin and tropomyosin on the one hand and myosin on the other. In the communication which follows, the results of such an investigation are described together with those of complementary morphological and chemical studies under conditions in which myosin was the main component to be extracted from the myofibrils.

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METHODS

Preparation and extraction of myofibrils. Myofibrils were prepared and extracted by the methods described in the preceding paper (Perry & Corsi, 1958). The stock pyrophosphate solution contained 0·1 M-$Na_4P_2O_7$, mM-$MgCl_2$, 10 mM-KH_2PO_4, and N-HCl (approx. 83 ml./l.) was added to bring the final pH to 7. Suspensions extracted with pyrophosphate were centrifuged for 15 min. at 14 000 *g*, and those extracted with 5 mM-acetate pH 4·3 for 60 min. at 105 000 *g*. The clear supernatants were used for adenosine triphosphatase (ATPase) and actomyosin assays.

Assay of myosin adenosine triphosphatase. The ATPase activity of extracts and suspensions was determined in 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4, 0·25 M-KCl, 5 mM-$CaCl_2$ and 5 mM-adenosine triphosphate (ATP). Incubations were carried out at 20° for 5 min. with three different concentrations of enzyme within the linear-response range. In the extraction with 5 mM-acetate buffer, pH 4·3, and with pyrophosphate solutions the whole suspensions and extracts were dialysed at 0° for 2 days against a solution containing 0·5 M-KCl and 0·039 M-borate, pH 7·1, which was changed several times. The percentage of myosin extracted from the myofibril was taken as 100 times the ATPase activity/ml. of the extract divided by the ATPase activity/ml. of the whole suspension.

Actomyosin test. The extract was dialysed for 2 days against a frequently changed solution containing 0·5 M-KCl and 0·04 M-phosphate buffer, pH 7·2. (Efficient dialysis after pyrophosphate extraction was essential to remove the last trace of pyrophosphate, which would dissociate any actomyosin present.) Viscosities were measured at 0° with an Ostwald viscometer (time of flow for water, 60 sec.) in the presence and absence of 0·5 mM-ATP (Bailey & Perry, 1947). A fall in viscosity on the addition of ATP indicated the presence of actomyosin.

Contraction test. Myofibrillar residues, washed free of the extraction medium, were treated with solutions containing 0·025–0·1 M-KCl, 5 mM-ATP and 5 mM-$MgCl_2$, with the pH adjusted if necessary to 7 with dilute alkali. Intact myofibrils contracted violently under these conditions. The test was either carried out by irrigating with the appropriate solution, the myofibrils being kept under observation on the microscope slide, or by inducing contraction in a small test tube and then examining the suspension microscopically.

Microscopic technique. All observations were carried out with the Cooke, Troughton and Simms Ltd. phase-contrast microscope and an oil-immersion achromatic objective, N.A. 1·3, and a ×15 eyepiece. A green filter was used for photography.

RESULTS

Characteristics of myofibril preparations

From fresh muscle in 0·025 M-KCl–*borate.* When prepared in this medium usually about 30 % of the myofibrils showed reversal of striation; on occasions the proportion of reversely striated myofibrils was even higher (Pl. 1: 1–4). In the majority of the myofibrils the I and A bands were readily recognized, the I band was usually relatively much shorter than the A band and in most cases the sarcomere length ranged from 1·9 to 2·3 μ. The H space, defined as an area of lower optical density in the centre of the A band of myofibrils possessing discrete A and I bands, was usually present and corresponded to one-third or less of the A band. Very occasionally the H space was not visible and a dark line which probably corresponded to the M line was visible in the centre of the A band (Pl. 1: 2). This dark line was never observed when the H space was apparent.

*From fresh muscle in ethylenediaminetetra-acetate–*KCl. If the KCl concentration of the borate medium used for the preparation of myofibrils was increased to 0·1 M many more of the myofibrils were reversely striated. If 5 mM-ethylenediaminetetra-acetate (EDTA) was added to 0·1 M-KCl and the pH adjusted to 7 (see Perry & Corsi, 1958), with very few exceptions all myofibrils showed distinct I and A bands (Pl. 1: 5–8) and the sarcomere length ranged from 1·7 to 2·5 μ (average length 2·2 μ). Although reversal of striation was only rarely observed there was a tendency for myofibrils prepared in EDTA to aggregate in suspension. Usually the A band was longer than the I, but frequently myofibrils of sarcomere length 2 μ or less, with the I longer than the A band, could be seen (Pl. 1: 6). The H space was apparent in practically all myofibrils and generally occupied one-third or less of the A band. In some cases it represented one-half or more of the A band (Pl. 1: 7, 8) but in these cases there was no obvious increase in length of the I band. In our preparations there was no clear correlation between the width of the H space and the length of the I band on the one hand and the sarcomere length on the other as demanded by the model of Hanson & Huxley (1955).

From glycerol-treated muscle in 0·1 M-KCl. Excellent myofibrils with distinct I and A bands were readily obtained from glycerol-treated muscle which had been stored for 2–3 weeks at −10°. (Pl. 1: 9, 10). If the glycerol-treated muscle was stored for shorter periods some reversal of striation occurred during the preparation, due no doubt to the endogenous ATP which was still present. The A band was usually longer than the I, and the H space was present in practically all myofibrils. On some occasions it was very narrow and only very rarely could not be recognized at all. Usually when the I band was longer than average the H space was likewise wider, but in our preparations there appeared to be exceptions to this rule.

It seems worthy of note, particularly in view of some of the other findings presented here, that in myofibrils isolated from both fresh and glycerol-treated muscle the Z line varied considerably in density and thickness. Usually the appearance was characteristic for a given myofibril (Pl. 1: cf. 5 with 3 or 7). A number of other authors (e.g. Cajal, 1888; Bennett & Porter, 1953) have commented on the variation of appearance of the Z line in fixed and stained preparations.

From the study of a large number of preparations obtained from both fresh and glycerol-treated muscle the A band did not appear to be constant in length. Sarcomeres with discrete I and A bands ranged in length from 1·7 to 3·0 μ and in general the longer the A band the greater the sarcomere length.

Extraction of components other than myosin from the myofibril

Low ionic strength, pH 7·1–8·3. On prolonged extraction of fresh myofibrils with 0·078 M-borate, pH 7·1, a fraction representing about 25 % of the total protein and consisting mainly of tropomyosin and actin was removed (Perry & Corsi, 1958). In Pl. 2: 11 and 12, which illustrate borate-extracted myofibrils, it can be seen that the Z line has disappeared and the I band also appears empty, although sufficient material remains to hold the A bands together along the myofibril. The region which corresponded to the H space in extracted myofibrils appeared less dense and usually wider than the original H space. Borate-extracted myofibrils still contracted on addition of ATP, although slowly, and the contracted material appeared disorganized.

When glycerol-treated myofibrils were extracted with borate the Z line was much more resistant; it usually survived 20–50 days of treatment, although it often appeared somewhat granular in appearance after such periods. The I band of glycerol-treated myofibrils was likewise more resistant to borate extraction than that of fresh myofibrils. In the former, strands of material would be quite clearly distinguished in the I band after prolonged borate extraction (Pl. 2: 13).

More rapid and exhaustive extraction of tropomyosin and actin was achieved with 5 mM-tris buffer, pH 7·7, (Perry & Corsi, 1958). After extraction under these conditions the picture was similar to that obtained with borate, the edges of the A band being the only prominent features of the original myofibril which were apparent (Pl. 2: 14). These edges were thinner in most cases than the original area bounded by the junction of the I and A bands and the edge of the H space. The I band was completely empty and in many cases it appeared to have collapsed to give the appearance illustrated in Pl. 2, 14 and 15. On continued extraction at pH 7·7 and with shorter periods at pH 8·3 the myofibrillar structure disintegrated. Myosin did not pass into solution under these conditions although about 35 % of the total protein was extracted.

When 35 % of the total protein had been extrated at pH 7·7 application of ATP to the washed residue produced a rather slow but obvious contraction, which suggested that some actin was still present in the residue.

Low ionic strength, pH 6·45. When fresh myofibrils were treated with 5 mM-phosphate buffer, pH 6·45, actin, tropomyosin and component *C* passed into solution, although much less protein was extracted than at higher pH values. The I band appeared to have been selectively extracted, for the Z line disappeared and the general density of the I band was much reduced (Pl. 2: 16–18). On the other hand, little change was apparent in the A band. In some preparations the I bands were so weakened that the myofibril broke up into individual A bands (Pl. 2: 18).

Extraction by procedures removing mainly myosin

Acetate buffer, pH 4·3. Little morphological change could be detected in myofibrils stored for 10 days in 5 mM-acetate buffer at pH 5·2. At pH 4·3 rapid extraction and marked structural changes could be observed within a matter of minutes when extraction was carried out by irrigation on the

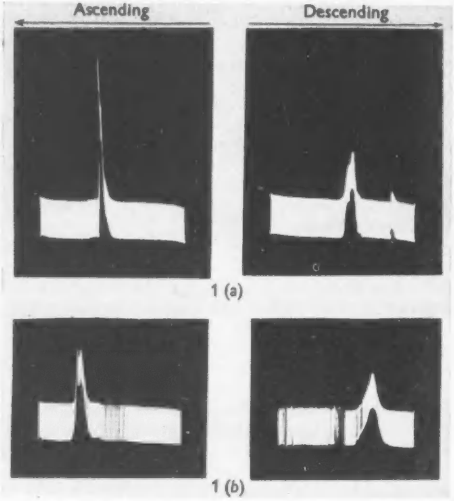


Fig. 1. Electrophoresis diagrams of myosin-containing extracts from myofibrils prepared from fresh muscle. Conditions: 0·25 M-KCl, 0·05 M-potassium phosphate buffer, pH 6·8. Preparation numbers refer to those listed in Table 1. (*a*) Myofibrils (174 FV, EDTA-prepared) dialysed against 5 mM-acetate buffer, pH 6·45, for 18 hr. Extract represented 63·9 % of total protein and contained tropomyosin equal to at least 2 % of total myofibrillar protein; actin present. Boundaries photographed after 720 min. (*b*) Myofibrils (177 FV, EDTA-prepared) extracted for 20 min. with 2 vol. of pyrophosphate solution. Extract represented 56·6 % of total proteins and contained tropomyosin equal to at least 1 % of total myofibrillar protein; actin present. Boundaries photographed after 900 min.

EXPLANATION OF PLATES 1–3

Plate 1

1. Myofibrils 157F isolated from fresh muscle in 0·025 M-KCl–borate and examined after 2 days. Normal myofibril with I, A, H and Z bands clearly visible and reversely striated myofibril.

2. Preparation as for no. 1. Myofibril with M line and a partly contracted myofibril (sarcomere = 1·5 μ) with pronounced Z line and a double-line system (A band?) in the centre of sarcomere.

3. Preparation as for no. 1. Myofibril with thick Z line.

4. Myofibrils 158F isolated from fresh muscle in 0·025 M-KCl–borate and examined immediately. Slightly shortened myofibril (sarcomere = 1·6 μ) with prominent Z bands and double-band system (A band?) in the centre of sarcomere (cf. no. 2).

5. Myofibrils 174FV isolated in 0·1 M-KCl–borate with 5 mM-EDTA and examined immediately. Typical myofibril.

6. Preparation as for no. 5. Myofibril of sarcomere length about 1·8 μ with broad I and comparatively narrow A band with H space.

7. Preparation as for no. 5. Myofibril with broad H space, relatively narrow I and faint Z line. Sarcomere length = 2 μ.

8. Myofibrils 162FV isolated in 0·1 M-KCl–borate–EDTA. Examined immediately. Wide H space and faint Z line.

9. Myofibrils 159G isolated in 0·1 M-KCl from glycerol-treated muscle. Dark line in the centre of A probably corresponding to the M line.

10. Myofibrils 154G isolated in 0·1 M-KCl from glycerol-treated muscle. Typical example showing A, I, H and Z bands.

Plate 2

11. Myofibrils 150F isolated in 0·025 M-KCl–borate. Extracted for 28 days in 0·078 M-borate, pH 7·1. 25% N extracted.

12. Myofibrils 129F isolated in 0·025 M-KCl–borate. Extracted for 88 days with 0·078 M-borate, pH 7·1. 24% N extracted.

13. Myofibrils 154G isolated in 0·1 M-KCl from glycerol-treated muscle. Extracted for 31 days with 0·078 M-borate, pH 7·1. Note remnants of I band and Z line.

14. Myofibrils 160F isolated in 0·025 M-KCl–borate. Extracted for 123 days with 5 mM-tris buffer, pH 7·7.

15. Myofibrils 169FV isolated in 0·1 M-KCl–borate–EDTA. Extracted for 7 days in 5 mM-tris buffer, pH 7·7. 29% N extracted.

16. Myofibrils 168FV isolated in 0·1 M-KCl–borate–EDTA. Extracted for 17 days with 5 mM-phosphate buffer, pH 6·45. 11·6% total N extracted.

17. Preparation and extraction as for no. 16.

18. Preparation and extraction as for no. 16. Myofibril breaking up into separate A band fragments.

Plate 3

19*a–c*. Myofibrils 174FV isolated in 0·1 M-KCl–borate–EDTA. Progressive extraction of the same myofibril by irrigation with 5 mM-acetate buffer, pH 4·3. Final stage corresponded to extraction of 60–70% of total protein.

20. Myofibrils 168F isolated in 0·1 M-KCl–borate–EDTA. Extracted for 4 days with 5 mM-acetate buffer, pH 4·3. Note granular Z band.

21. Myofibrils 174FV isolated in 0·1 M-KCl–borate–EDTA. Extracted for 20 min. with 3 vol. of pyrophosphate, pH 7. This is the appearance on complete extraction with pyrophosphate. Note gaps in the centres of sarcomeres which are longer than the original H space.

22. Preparation and extraction as for no. 21.

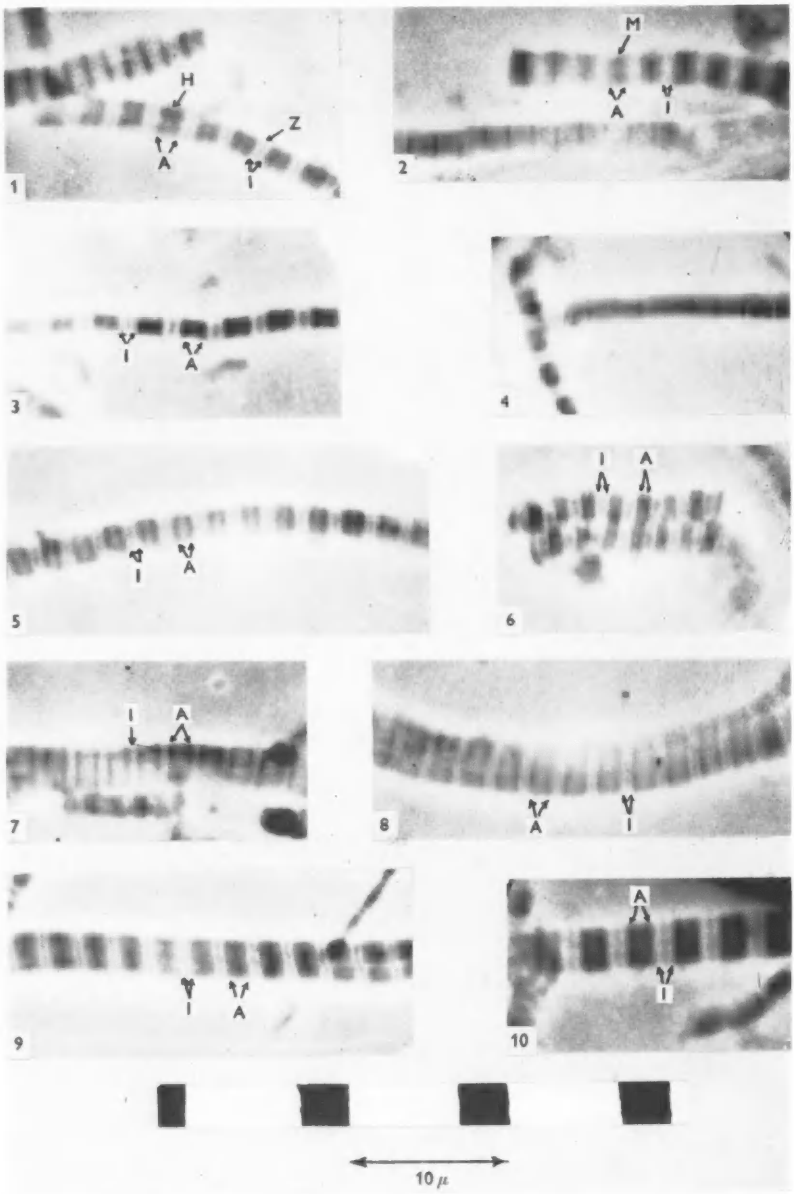
23. Intermediate stage of extraction described in no. 21.

24. Myofibrils 178G isolated from glycerol-treated muscle in 0·1 M-KCl–borate. Extracted for 2–3 hr. with 2 vol. of pyrophosphate. Note 'ballooning' effect in A band area, which was sometimes observed during pyrophosphate extraction.

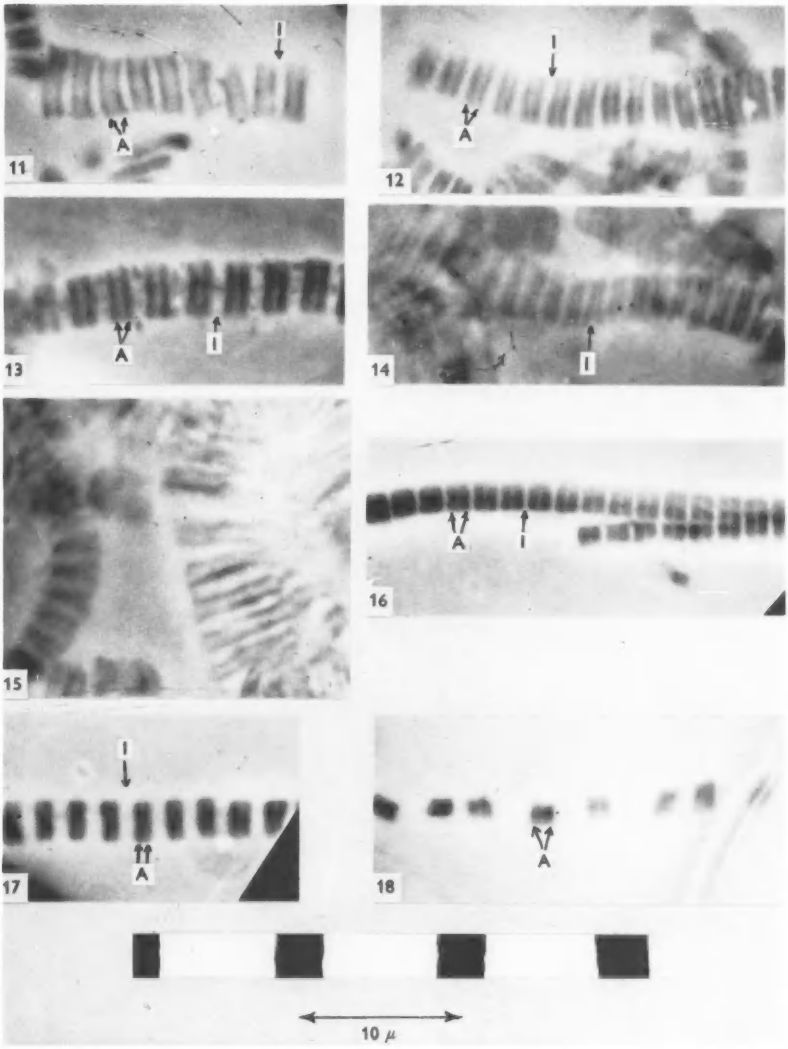
25. Preparation and extraction as for no. 24. Final appearance of 20–30% of glycerol-treated myofibrils.

26. Preparation as for no. 24. Extracted with 2·4 vol. of pyrophosphate. Sarcomeres (length = 1·8 μ) showing broad gaps.

(*Facing p.* 14)



A. CORSI AND S. V. PERRY—SOME OBSERVATIONS ON THE LOCALIZATION OF MYOSIN, ACTIN AND TROPOMYOSIN IN THE RABBIT MYOFIBRIL



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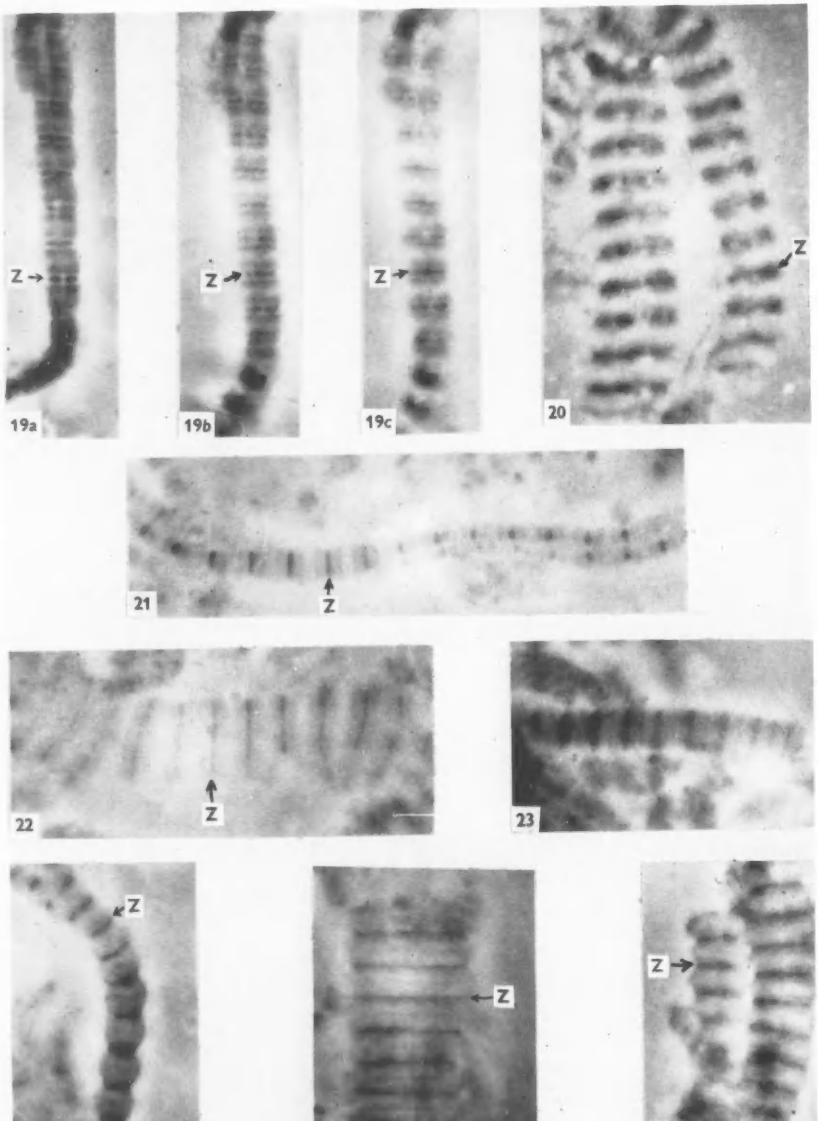

A. CORSI AND S. V. PERRY—SOME OBSERVATIONS ON THE LOCALIZATION OF MYOSIN, ACTIN AND TROPOMYOSIN IN THE RABBIT MYOFIBRIL

microscope stage, but the process could be appreciably slowed down by dialysing myofibril suspensions against the buffer. After prolonged extraction 60–70% of the total N passed into solution. Such extracts contained mainly myosin, together with some actin and tropomyosin. On electrophoresis in 0·25 M-KCl and 0·05 M-phosphate buffer, pH 6·8, two main peaks which probably corresponded to L-myosin and actomyosin were obtained (Fig. 1*a*). Under the conditions of the actomyosin test the relative viscosity of the extract studied in Fig. 1*a* was 3·36; in the presence of ATP it was 3·27. Although it can be concluded that actin is present, at first sight these figures suggest that the amount is small. This is not necessarily so as the ability of myosin to form actomyosin, which can be demonstrated viscometrically, is considerably reduced by exposure of myosin to pH values below 5 (Perry, unpublished work). Likewise, exposure to pH 4·3 considerably reduced the myosin ATPase activity, but preparations were sufficiently active after 2–3 days for satisfactory assays to be carried out. The results obtained are summarized in Table 1 and suggest that when 64–67% of the protein is in solution all the myosin has been extracted.

When the extraction of individual myofibrils was observed on the microscope stage the first effect of 5 mM-acetate, pH 4·3, was to decrease the density of the A band. This band was eroded away from the centre, a process which was sometimes accompanied by swelling (Pl. 3: 19*b*, *c*). At about the same time the Z band appeared much thicker and more dense until it was the most striking feature of the myofibril. Later, in the middle of the A band a gap appeared which was broader than the original H space, with the result that there could be seen in the fragments which remained a rather thick and dense Z line and thin bands at the edges of the I band area. These bands probably corresponded to the edges of the A band (Pl. 3: 19*a*–*c*). On prolonged extraction the area on each side of the Z line tended to become more even in density, although it was obvious that the density of this area appeared higher than that of the original I band (Pl. 3: 20). It may be suggested that the increase in relative darkness of the Z line and the I band area compared with the original unextracted myofibril is an optical effect due to the extraction of the strongly refracting material from the neighbouring A band. This explanation does not seem entirely satisfactory for the apparent increase in thickness of the Z line and it is possible that some translocation of protein may have occurred.

Pyrophosphate, pH 7. When fresh myofibrils were extracted with 2–4 vol. of $Na_4P_2O_7$ solution, pH 7 (see Methods), 56–60% of the total N rapidly passed into solution and little further N was extracted by more prolonged treatment. On electrophoretic analysis two main components, which probably corresponded to L-myosin and actomyosin, were obtained (Fig. 1*b*). The presence of actomyosin was confirmed by the drop of viscosity obtained on the addition of ATP to the solution of the extract. After dialysis against the medium used for the actomyosin test the extract studied in Fig. 1*b* had a relative viscosity of 2·74, which was reduced to 2·23 on addition of ATP. ATPase assay of dialysed whole suspensions and extracts indicated that 95–100% of the myosin was extracted.

Chemically the fraction extracted by the pyrophosphate solutions was similar with both fresh and glycerol-treated myofibrils, although in the latter there was some suggestion that less actin was extracted. After dialysis of pyrophosphate extracts of glycerol-treated myofibrils against 0·078 M-borate

Table 1. *Nitrogen and adenosine triphosphatase extracted from rabbit myofibrils by 5 mM-acetate buffer, pH 4·3, and pyrophosphate solutions*

In the extraction with pyrophosphate the figures in the second column represent the volumes of solution added to 1 vol of myofibril suspension. Preparations 178G and 179G were made in 0·1 M-KCl–borate from glycerol-treated muscle; the others were made from fresh muscle in the same medium, with EDTA present during initial homogenization.

Preparation no.	Extractant	Concn. (mg. N/ml.)	Extraction time	N extracted (%)	ATPase activity (μg. of P/min./ml.) Whole	Extract
177FV	Pyrophosphate (2 vol.)	1·30	20 min.	57·8	56·6	60·0
177FV	Pyrophosphate (4 vol.)	0·771	20 min.	56·7	36·8	33·9
178G	Pyrophosphate (2·4 vol.)	1·027	4 hr.	59·4	36·5	38·7
179G	Pyrophosphate (2·4 vol.)	1·096	4 hr.	56·8	45·4	44·0
173FV	0·1 M-KCl–borate	4·16	40 hr.	—	389	(Nil)
	5 mM-Acetate, pH 4·3*	—	40 hr.	38	159	100·4
174FV	5 mM-Acetate, pH 4·3	1·157	18 hr.	64	39·6	43·8
			43 hr.	67	24·4	23·4

* Slow extraction by dialysis.

buffer, pH 7·1, to precipitate myosin and actomyosin, tropomyosin could not be detected in the protein remaining in solution, whereas with fresh myofibrils an amount corresponding to at least 2% of the total N was present.

The appearance of myofibrils after complete extraction with pyrophosphate (Pl. 3: 21, 22) was very similar to that obtained after exhaustive extraction with acetate buffer, pH 4·3, as indeed the chemical evidence would indicate.

Nevertheless, important differences were apparent in the form the extraction took. Initially the H space disappeared and a dense line narrower than the original A appeared midway between the Z lines. Later this new band lost intensity so that the space between the Z lines acquired a more even density, apparently greater than that of the original I band. Finally the centre of the sarcomere, representing most of what was originally the A band, was extracted practically completely and the myofibrils appeared very similar to those shown in the photographs published by Hanson & Huxley (1955) after pyrophosphate extraction, and to those obtained on extraction with 5 mM-acetate, pH 4·3 (Pl. 3: 19, 20).

More prolonged extraction of glycerol-treated myofibrils with pyrophosphate solutions was required to obtain the same morphological picture as was obtained with fresh preparations (Pl. 3: 24–26). Even after 12 days some 20–30% of the myofibrils had a thin line midway between the Z lines (Pl. 3: 25). Some myofibrils had also a thin line on each side of the Z, so that, in addition to the latter, three lines could be seen in each sarcomere. A similar pattern was also sometimes observed during the extraction of fresh myofibrils (Pl. 3: 23).

After extraction of myofibrils and subsequent washing, first with pyrophosphate solution and then with 0·1 M-KCl–borate, in some cases it was possible to induce limited contraction by the addition of ATP. The effect was much less striking than that obtained with fresh myofibrils and indicated that myosin was still present in the residue. In view of the results of the ATPase experiments this must represent a small proportion of the whole of the myosin present in the intact myofibril.

DISCUSSION

Consideration of the results of the morphological studies in conjunction with the chemical investigations described in this and the preceding paper suggest that the following conclusions can be drawn about the localization of proteins within the myofibril.

Myosin

When the I band is extracted so that optically dense material cannot be detected by the phase-contrast microscope, conditions which lead to the collapse of the I band or the breaking away of individual A bands, no myosin ATPase passes into solution. It can therefore be concluded that myosin is not present in the I band in any significant amount. Likewise it can also be concluded that this protein is absent from the Z band as it appears in uncontracted myofibrils, for when the Z band disappears myosin ATPase does not pass into solution. It follows that myosin is localized entirely in the A band and in this respect our results confirm and extend the findings of Hasselbach (1953), Hanson & Huxley (1953) and those more recently obtained by Finck, Holtzer & Marshall (1956), but do not support the views of Szent-Györgyi, Mazia & Szent-Györgyi (1955) who consider that there is a more even distribution of myosin along the myofibril.

It may be of interest to point out that the residue remaining after extraction of actin, tropomyosin and component *C*, and which represents about 65% of the total protein, probably contains at least 80% of myosin, but at the moment we feel that there is insufficient evidence to decide finally whether the dense edges of the A band, which are the main features of these extracted myofibrils, represent an accumulation of myosin or some other protein such as residual actin and tropomyosin.

Actin and tropomyosin

These proteins are taken together for we have no evidence to suggest that they are independently localized. On the contrary, the chemical evidence can be interpreted to indicate that they are associated *in situ*, for under a wide variety of conditions these proteins were extracted in fairly similar proportions. Both proteins were obtained when the I band was preferentially extracted at pH 6·45, and at higher pH values when they were also extracted from the A band. On exhaustive extraction at higher pH values the amount obtained was about thrice that extracted at pH 6·45. As normally the A band of myofibrils at equilibrium length is about twice as long as the I band these yields would fit in well with the view that actin and tropomyosin are evenly distributed along the sarcomere in the resting myofibril. Irrespective of the validity of this latter argument it can be concluded that actin and tropomyosin occur in both the I and A bands. Our investigations provide no evidence pointing to the absence of actin in the H space; the results suggest that extraction procedures which decrease the density of the H space extract actin. On treatment with pyrophosphate solutions fresh myofibrils were readily extracted to give the structures illustrated in Pl. 3: 20 and 21, which are similar to those described by Hanson & Huxley (1955). In our preparations the gaps produced in the centre of the sarcomere are wider than the H spaces and cannot be considered to represent the

original H spaces. Careful study of the figures published by Hanson & Huxley (1955) suggests that this fact is also true under their conditions of extraction. Furthermore, as analysis of the protein extracted by pyrophosphate solutions clearly indicates the presence of actin, it cannot be concluded from these experiments that the gap seen in pyrophosphate-extracted sarcomeres corresponds to a break in continuity of actin filaments in the intact myofibril. Continuity of the filaments remaining after extraction of myosin has been demonstrated by Hasselbach (1953).

Hasselbach (1953) has presented evidence that the Z band can be dissolved from the myofibril under conditions of higher ionic strength (e.g. at $I = 0{\cdot}6$). Under our conditions of extraction with low-ionic-strength buffers the Z band likewise disappears, and if it passes into solution then the protein arising from it should be found in the soluble fraction. In view of the fact that the amount of component *C* present can be much reduced by more extensive washing of the myofibril without the disappearance of the Z band, it seems unlikely that component *C* arises from this structure. Undoubtedly the main components of the soluble fraction are actin and tropomyosin but it is possible that this fraction contains small amounts of other proteins which are not readily detected by electrophoretic methods. At this stage the protein nature of the Z band must remain unresolved.

According to the model of the myofibril proposed by Huxley & Hanson (1954) and Huxley & Niedergerke (1954) the H space disappears when the sarcomere length falls below $2\,\mu$. Such a model of myofibrillar structure is very difficult to reconcile with the appearance of many of the myofibrils isolated from fresh muscle. If the H space is defined as described above, it can readily be recognized in fresh myofibrils of sarcomere length less than $2\,\mu$ (see Pl. 1: 6). Such myofibrils are not unusual, and the H space is frequently relatively wide (more than half of the A band). This model likewise demands that actin be absent from the H space, but the evidence presented here gives no indication of this; on the contrary it suggests that either actin or tropomyosin or both are present in the H band region of the myofibril.

SUMMARY

1. Extraction of actin, tropomyosin and component *C* from isolated rabbit myofibrils was accompanied by a disappearance of the Z line, almost complete disappearance of material from the I band and a fall in density and widening of the H space.

2. Pyrophosphate solutions and 5 mM-acetate buffer, pH 4·3, extracted practically all the myosin together with some actin and tropomyosin. On exhaustive treatment with these solutions the A band was removed from the sarcomere almost completely.

3. In myofibrils isolated from fresh muscle the H space was apparent in sarcomeres of a wide range in lengths. No obvious correlation was apparent between the lengths of the I band and H space.

4. It is concluded that myosin is absent from the I band and Z line and that actin and tropomyosin are present in both I and A bands.

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Acid-Soluble Phosphates in the Lens

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Pirie, van Heyningen & Flanders (1955) showed that the concentrations of certain acid-soluble phosphates in the lens, when estimated by the enzymic method of Slater (1953), were much lower than the concentrations reported by Nordmann & Mandel (1952), who used the barium-fractionation method of LePage (1948, 1949). This method has, until recently, been the accepted and standard one for estimation of acid-soluble phosphates. Using the enzymic method we found large deficits in creatine phosphate, hexose phosphates and phosphoglyceric acid. It seemed doubtful whether creatine phosphate was present in measurable quantity and the sum of the hexose phosphates and phosphoglyceric acid amounted to only 12–40 μmoles of P/100 g. of lens when estimated enzymically, whereas the chemical estimation had given about 400 μmoles of P/100 g. of lens in this form. Similar discrepancies were found in the analyses of the acid-soluble phosphates of rat kidney and liver, but not of those of rat muscle, where enzymic and chemical analyses agreed. LePage's analysis of the rat muscle has also been confirmed recently by Threlfall (1957), using a method based on chromatography.

The enzymic method can claim greater specificity than the chemical one and it seemed likely that it gave truer values. This implied that the nature of a considerable part of the organic acid-soluble phosphates of lens and of other tissues was still unknown. We have therefore analysed the acid-soluble phosphates of lens by chromatographic and other methods in an attempt to identify further substances and narrow the gap between total phosphorus and the phosphorus in known compounds.

The analysis which we report has not uncovered any compound not already known in other tissues but it has radically altered our conception of acid-soluble phosphates in the lens, and this may make necessary a reorientation of views of lens metabolism. We have examined the lens of both rabbit and ox.

METHODS

Animals. Cattle (calves of 1–7 days old and older beasts) and rabbits of known age were used. Cattle eyes were removed as soon as possible after death and brought from the slaughterhouse in ice; the lenses were removed and either used immediately or kept frozen. Hereafter the calves and older beasts will be referred to as calves and cattle respectively. Rabbits were killed with intravenous Nembutal and the lenses were chilled and used at once.

Lens extracts. A calf lens weighs about 1 g., a cattle lens about 2–2·5 g., and the lens of a rabbit about 0·3 g. at 3 months of age and about 0·6 g. when 2 years old or more. About 20 g. of calf or cattle lenses was used to make an extract and about 5 g. of rabbit lenses. Three types of extract were used:

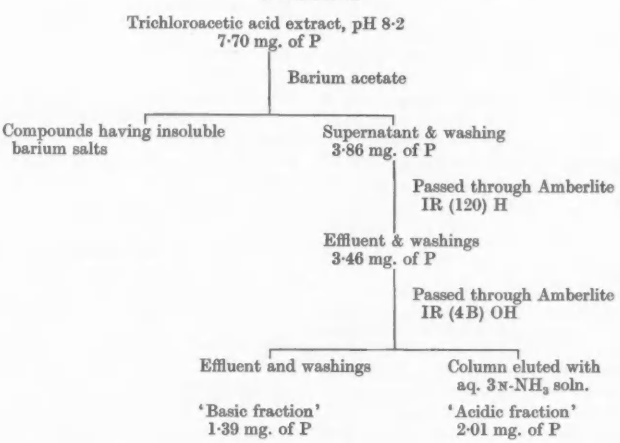
(i) Trichloroacetic acid (TCA) followed by barium precipitation. It is essential that trichloroacetic acid extracts of lens be fractionated and deionized for chromatographic separation of the phosphate compounds. The following procedure was found satisfactory. The lenses were weighed, ground in three or five times their weight of ice-cold TCA (8%, w/v), stored at 0° for 30 min. and centrifuged at 12 000 *g* for 15 min. at 0°. Most of the TCA was removed by three extractions each with 3 vol. of ether. The extract was then made just pink to phenolphthalein (pH 8·2) with dil. aq. NH_3 soln., and 1 ml. of saturated barium acetate was added for each 60 ml. of TCA extract. After 15 min. at 4° the barium precipitate was centrifuged down; it was washed according to the method of LePage (1949) and the extract and washing were combined. The water-insoluble barium precipitate was kept frozen.

The combined extract and washing were passed through a column of Amberlite IR(120)H (16–50 mesh), 20 cm. long and 1 cm. diameter, at the rate of about 0·5 ml./min., and washed through with a volume of water equal to about four times the volume of the combined extract and washings. The effluent was collected and, after removal of a small volume for the estimation of total P, passed through a column of Amberlite IR(4B)OH (20–50 mesh) (Ansell & Norman, 1956). The effluent from the second column was concentrated at 40–50° *in vacuo*, evaporated to dryness in a desiccator and made up to a suitable volume with water. This was called the 'basic fraction'. The more acidic compounds which were retained by the Amberlite IR(4B)OH column were eluted from it with aq. 3N-NH_3 soln. This eluate was taken to dryness, made up to a known volume and called the 'acidic fraction'.

The barium precipitate was suspended in a little water in a centrifuge tube and shaken with sufficient Amberlite IR(120)H to remove all the barium and thus bring the phosphorus-containing compounds into solution. The Amberlite was centrifuged down and washed twice with water. The combined extract and washings were neutralized with dil. aq. NH_3 soln., concentrated *in vacuo* and dissolved in a known volume of water. The treatment with Amberlite was done quickly and at 0° to prevent the breakdown of adenosine triphosphate (ATP) and other phosphorus-containing compounds.

Thus the TCA extract of lens was divided into three fractions, a 'basic fraction' and an 'acidic fraction', both of

Table 1. *Fractionation of trichloroacetic acid extract of calf lens*



which contain compounds having water-soluble barium salts, and a fraction containing compounds having water-insoluble barium salts. Table 1 gives the results of an experiment with calf lens. About half the total phosphate compounds have water-insoluble barium salts. Ninety per cent of the P in the extract containing the soluble barium salts was recovered in the effluent from the Amberlite IR(120)H column. On one occasion the small amount of organic P remaining on this column was eluted with aq. 3N-NH_3 soln. and examined chromatographically. It appeared to contain traces of a number of the compounds subsequently detected in the three analysed fractions and it was therefore assumed that, had the column been more exhaustively washed, all the P would have been recovered in the effluent.

(ii) Boiling dilute acetic acid. This method is a modification of that used by Campbell & Work (1952). Frozen or ice-cold lenses were weighed and dropped into five times their weight of boiling 0·07% (w/v) acetic acid. They were stirred and broken up with a glass rod and after 3 min. the boiling suspension was poured into a mortar and the lenses were thoroughly ground. The suspension was then boiled for a further 2 min., cooled and its volume measured, and centrifuged at 0° at 12 000 *g* for 20 min. The clear colourless extract was poured off and its volume measured. The pH was about 6·3.

The possible advantage of this type of extraction is that acid-labile substances may be preserved, and that phosphate esters will not be liberated from phospholipids (Dawson, 1955*a*, *b*). There is evidence of breakdown of phospholipid during trichloroacetic acid extraction of tissues (Ansell & Norman, 1953). A disadvantage of this method of extraction, when applied to the lens, is that the extract still contains a considerable amount of protein which has to be removed during the subsequent fractionation.

(iii) Ethanol. Extraction of lenses in 80% ethanol at room temperature (Shaw, 1955) was used occasionally, as an alternative to trichloroacetic acid extraction, in the estimation of phosphorylethanolamine in lens.

Paper chromatography. Whatman no. 1 paper was washed with 2N-acetic acid followed by six successive lots of water. Both descending and ascending chromatography were used, usually the former.

Solvents. Phenol–aq. NH_3 soln. (sp.gr. 0·890 diluted with water, v/v to 0·3%); 10:28 (w/v). Phenol–water, 100:28 (w/v). Butanol–acetic acid–water, 40:9:20 (Waley, 1956). Propan-1-ol–aq. NH_3 soln. (sp.gr. 0·890)–water, 60:30:10 (Hanes & Isherwood, 1949). *iso*Propyl ether–formic acid (90%), 90:60 (w/v) (Hanes & Isherwood, 1949). *iso*Butyric acid–aq. N-NH_3 soln., 100:60 (Krebs & Hems, 1953). Propan-2-ol (680 ml.)–HCl (176 ml.)–water (to 1 l.) (Wyatt, 1951). 2-Methylpropan-2-ol (700 ml. at 26°)–constant-boiling HCl (132 ml.)–water (to 1 l. at 20°) (Smith & Markham, 1950). Butanol–water–formic acid (98–100%), 77:13:10 (Markham & Smith, 1949). Butanol–diethylene glycol–water, 4:1:1 (Levine & Chargaff, 1951).

Detection and determination of substances after chromatography. Compounds containing P were detected by dipping the papers in the molybdate reagent of Burrows, Grylls & Harrison (1952), and exposing the papers when thoroughly dry to direct sunlight or, when this was impossible, to light from an Osira ultraviolet lamp. Phosphate was estimated quantitatively by ashing the paper according to the method of Hanes & Isherwood (1949), and determining the resulting orthophosphate (P_i) by the method of Berenblum & Chain (1938). Choline was located by the method of Levine & Chargaff (1951) or Munier (1951), glycerol by the silver nitrate method of Trevelyan, Proctor & Harrison (1950) and amino compounds with the ninhydrin reagent of Wiggins & Williams (1952). Nucleotides were located on the chromatograms by their ultraviolet absorption with a Hanovia 'Chromatolite' (Hanovia Ltd., London) and their positions ringed in pencil. On some occasions the absorbing areas were extracted in 0·01N-HCl, and the absorption was measured in a Beckman spectrophotometer.

Paper electrophoresis. Horizontal strips of Whatman no. 1 or 3MM paper between sheets of polythene and glass were used (Kunkel & Tiselius, 1951). The potential difference along the paper was about 10v/cm. Borate buffer, pH 9 (7·63 g. of sodium borate, 0·62 g. of boric acid in 1 l. of water), pyridine–acetic acid buffer, pH 4 (50 ml. of acetic acid, 15 ml. of pyridine and 2·5 l. of water; Grassmann, Hannig & Plöckl, 1955) and ammonium formate (0·05M, pH 3·2) were used.

Other estimations. Phosphate was estimated by the method of Fiske & Subbarow (1925), choline by the reineckate method of Engel, Salmon & Ackerman (1954) and pentose by the adaptation of Ceriotti (1955) of the method of Barrenschein & Perham (1941). Propane-1:2-diol phosphate was estimated by the method of Huggins & Miller (1956).

Materials

Chromatographic markers. Glycerylphosphorylethanolamine (GPE), glycerylphosphorylcholine (GPC) and phosphorylcholine (PC) were gifts from Dr R. M. C. Dawson. Phosphorylethanolamine (PE) was made by the method of Outhouse (1937). Inosine mono-, di- and tri-phosphates (IMP, IDP, ITP) were gifts from Mr R. Hems. Other substances used as markers were obtained commercially. Acid phosphatase was made from potato by the method of A. Kornberg (unpublished work), the details being supplied by Dr B. L. Horecker.

RESULTS

The three fractions into which the lens extract was separated by method (i) were examined separately.

'*Basic fraction*'

Spots (5 μl.) of this fraction were superimposed on paper until about 35 μg. of P in all had been applied, and this was submitted to two-way chromatography (phenol–aq. NH_3 soln., propanol–aq. NH_3 soln.–water). The paper was treated first with ninhydrin and then with the phosphate-detecting reagents. The spots were cut out and their P content was measured, and it was shown that most of the P was accounted for in the five areas which stained blue with the molybdate reagent (Table 2).

Three ninhydrin-positive spots were found; of the three, two contained P and from their R_F values were presumed to be PE and GPE; the third, a very large spot, contained no P and had R_F values corresponding to taurine (Campbell & Work, 1952). Glutathione, other peptides and amino acids were retained on the Amberlite IR(120)H column (Campbell & Work, 1952).

The two areas which contained P but which were ninhydrin-negative were presumed to be PC and GPC, again from their R_F values in these solvents (Dawson, 1955*a*).

The streak of phosphorus-containing compounds from the origin was not further characterized.

The whole of the basic fraction which remained was applied as narrow bands across the width of several chromatographic papers and run in phenol–aq. NH_3 soln. overnight. About 300 μg. of P was loaded on to each paper. Narrow strips from the long edges of the paper were developed first with ninhydrin and then with molybdate, to locate the position of the various compounds. Three areas of paper were removed for elution, the first around R_F 0·26 containing the presumed PE, the second around R_F 0·67 containing the presumed GPE and the third around R_F 0·90 containing the presumed PC and GPC. The three eluates were all taken to dryness *in vacuo* and dissolved in a known volume of water.

Identification of phosphorylcholine and glycerylphosphorylcholine

(i) Identification of P and choline. The presence of P in the compounds had already been shown. To show the presence of choline (Dawson, 1955*a*) spots of the eluate were chromatographed in propanol–aq. NH_3 soln.–water, and the two phosphorus-containing areas were cut out and extracted with boiling water. Ammonium reineckate in methanol was added and the tubes were left in the refrigerator. After 1 hr. shining crystals of choline reineckate could be detected in both. No crystals appeared when pieces of paper adjacent to the spots were treated in the same way.

Table 2. *Analysis of two-way chromatogram of 'basic fraction' from calf lens*

See text for details. Solvents, phenol–water (A), followed by propanol–aq. NH_3 soln.–water (B). Approx. 38·5 μg. of P was applied to paper.

Nature of compound	Approximate R_F (A)	Approximate R_F (B)	Spot tests on chromatogram: Ninhydrin	Spot tests on chromatogram: Molybdate (for P)	P in spot (μg.)
Phosphorylethanolamine	0·26	0·17	+	+	2·8
Taurine	0·37	0·40	+	–	None
Glycerylphosphorylethanolamine	0·66	0·42	+	+	2·0
Phosphorylcholine	0·89	0·25	–	+	6·3
Glycerylphosphorylcholine	0·90	0·41	–	+	16·9
Unknown	Streak from origin		–	+	6·2
				Total	34·2

(ii) Cochromatography with synthetic markers. When spots of PC and of GPC were superimposed on spots of the eluted fraction from lens extract and these were chromatographed in phenol–aq. NH_3 soln., propanol–aq. NH_3 soln.–water or *iso*propyl ether–formic acid, no separation of the markers occurred and the R_F values of the phosphorus-containing spots agreed with expectation.

(iii) Paper electrophoresis with synthetic markers. Paper electrophoresis of the eluted lens fractions at pH 9 showed three phosphorus-containing spots. The first had not travelled and corresponded in position with the GPC marker. The second had moved 3·7 cm. to the positive pole. It was a very weak spot and was not identified. The third spot had moved 4·3 cm. to the positive pole and corresponded to the PC marker spot. Inorganic P moved about 13 cm. under these conditions.

(iv) Hydrolysis and identification of hydrolysis products. GPC is hydrolysed to choline and α-glycerophosphate by hydrolysis in N-HCl for 30 min. at 100°, whereas PC requires 24 hr. hydrolysis in 2N-HCl at 123° (Dawson, 1955*a*).

Part of the eluate was hydrolysed in N-HCl for 30 min. It was then evaporated to dryness *in vacuo*, dissolved in water, run on a two-dimensional chromatogram (phenol–aq. NH_3 soln., propanol–aq. NH_3 soln.–water) and sprayed for P. The spot corresponding to PC was unchanged but the spot corresponding to GPC had disappeared and a spot corresponding to α-glycerophosphate was now present.

A chromatogram of the unhydrolysed and hydrolysed lens fraction and of choline hydrochloride was developed in phenol–aq. NH_3 soln. and the paper sprayed to detect choline. There was a spot corresponding to choline after hydrolysis of the lens fraction, but not before.

Since the reineckate precipitation may not be specific for choline, the formation of choline on mild and severe hydrolysis of lens extracts was confirmed chromatographically. Butanol–acetic acid–water and butanol–diethylene glycol–water were used as solvents. In both solvents a spot having the same R_F value as choline was obtained and cochromatography with authentic choline gave a single spot.

Estimation of phosphorylcholine and glycerylphosphorylcholine in the lens

After two-dimensional chromatography of the 'basic fraction' of calf, cattle and rabbit lens (phenol–water, propanol–aq. NH_3 soln.–water) the spots were made visible with the molybdate spray, and the P in the spots was estimated. Table 3 shows that the calf lens contains 230–400 μmoles of P/100 g., as choline compounds, with rather more GPC than PC. Cattle lens contains 100–200 μmoles of P/100 g. as these compounds. The lens of the rabbit contains less. Similar values were obtained with the three types of extract. The level in the calf lens is comparable with levels found by Dawson (1955*a*, *b*) in other tissues.

Choline was estimated in lens extracts after short and prolonged periods of hydrolysis. Unhydrolysed extracts contained none. Table 3 gives

Table 3. *Phosphorylcholine (PC) and glycerylphosphorylcholine (GPC) in lens*

P was measured in chromatographic spots and choline was measured as the reineckate in hydrolysed extracts.

Type of extract	How measured	P (μmoles/100 g. of lens)		
		As PC	As GPC	As PC+GPC
Calf (ii)	P	87	—	—
(ii)	P	168	216	384
(ii)	Choline	194	210	404
(ii)	Choline	—	181	—
(ii)	P	100	129	229
	Choline	—	—	236
(i)	P	116	216	332
(i)	P	113	219	332
(i)	P	79	232	311
Cattle (ii)	P	64	132	196
	Choline	45	106	151
(ii)	P	32	76	108
	Choline	—	—	144
(i)	Choline	64	93	157
(i)	Choline	—	93	—
Rabbit (i) Age about 3 months	P	55	77	132
(ii) Age 5 months	P	45	—	—
(i) Age 7 months	P	45	32	77
(i) Age 7–9 months	P	55	—	—
(i) Age 9 months	P	87	10	97
(i) Age 2 years or more	P	45	23	68

the values for choline liberated by mild and strong hydrolysis, expressed as μmoles of P/100 g. of lens, on the assumption that all lens choline is in the form of PC and GPC. There is reasonable agreement between the values for P as GPC and PC expressed in this way and the values calculated from the P content of the spots which correspond to PC and GPC on a chromatogram of a lens extract.

Identification of glycerylphosphorylethanolamine

(i) Cochromatography with synthetic marker. Spots of the concentrated eluate containing the substance presumed to be GPE were superimposed on a spot of synthetic GPE, and chromatographed in phenol–water followed by propanol–aq. NH_3 soln.–water. The papers were treated with ninhydrin and it was found that no separation of the two substances had occurred.

(ii) Paper electrophoresis before and after hydrolysis. GPE is hydrolysed to glycerophosphate (GP) and ethanolamine by 60 min. hydrolysis at 100° in N-HCl. Part of the eluate was hydrolysed under these conditions, evaporated to dryness *in vacuo* over NaOH and dissolved in water.

Paper electrophoresis at pH 4 of the eluate before and after hydrolysis, with GP, ethanolamine and P_i as markers, gave the following results. Before hydrolysis the eluate gave one spot, containing P and ninhydrin-positive, which did not migrate. After hydrolysis this spot had almost disappeared and instead there was a ninhydrin-positive spot migrating to the cathode in the position of ethanolamine, and a phosphorus-containing spot migrating to the anode in the position of GP.

Identification of phosphorylethanolamine

(i) Cochromatography with synthetic markers. In the basic fraction of the lens extract, the compound presumed to be PE travelled in phenol–water at a rate considerably lower than that of taurine and this made possible the separation of these two compounds. A spot of eluted compound, superimposed on a spot of synthetic PE, did not separate from it when run in a two-way chromatogram (phenol–water, propanol–aq. NH_3 soln.–water).

(ii) Paper electrophoresis before and after hydrolysis. A sample of the hydrolysate was incubated for 18 hr. at 120° in 6N-HCl in a sealed tube, conditions known to hydrolyse PE. Paper electrophoresis at pH 4 of the eluate before and after hydrolysis gave the following results. Before hydrolysis the substance did not migrate, and gave positive reactions with the ninhydrin and phosphate reagents. After hydrolysis, spots of inorganic phosphate and ethanolamine appeared and the PE spot was greatly reduced in intensity.

Estimation of phosphorylethanolamine and glycerylphosphorylethanolamine in lens

These compounds were estimated in the same two-way chromatograms as those prepared for the estimation of PC and GPC. PE was also estimated in a similar way in extracts made by method (iii) (Table 4).

'Acidic fraction'

This fraction accounts for about 413 μmoles of P/100 g. of calf lens and 608 μmoles of P/100 g. of young-rabbit lens. A 5 μl. spot, containing about 10 μg. of P, was run in phenol–water, and the paper was treated with the molybdate reagent to locate phosphorus-containing areas. There was a considerable amount of P_i together with some organic phosphate, running between R_F 0 and 0·18, and a small fast-running phosphorus-containing spot (R_F 0·9) which subsequently was shown to be due to PC, which had been retained by the Amberlite IR(4B)OH column. The rest of the phosphorus-containing compounds were found on incineration in the part of the paper between R_F 0·18 and 0·5, but their presence was not always shown at this stage by the appearance of a blue colour with the molybdate reagent. In the calf lens the P in this area accounted for about 230 μmoles of P/100 g. of lens, the P_i for about 100 μmoles of P/100 g. of lens, and the PC for 30 μmoles of P/100 g. of lens.

The whole fraction was loaded in narrow bands along the width of chromatographic papers and chromatographed in phenol–water overnight. About 250 μg. of P was applied to each paper. The papers

Table 4. *Glycerylphosphorylethanolamine (GPE) and phosphorylethanolamine (PE) in the lens*

P was measured in chromatographic spots.

Type of extract		P (μmoles/100 g. of lens) As GPE	As PE
Calf (i)		32	35
(i)		40	34
(iii)		—	23
Rabbit (i)	Age about 3 months	29	13
(iii)	Age about 6 months	—	None detected
(i)	Age 7 months	24	None detected
(i)	Age over 2 years	None detected	None detected

were thoroughly dried and strips from each long edge cut off and stained for P. The area of the papers from the boundary of the area containing P_i (about R_F 0·18) up to R_F 0·5 was cut out, eluted with water, taken to dryness *in vacuo* and dissolved in water. A spot of this eluate containing about 10 μg. of P was run in propanol–aq. NH_3 soln.–water overnight. There were now found to be five distinct phosphorus-containing compounds in the fraction which reacted with molybdate giving a blue colour: a trace of P_i, R_F 0; three compounds absorbing strongly in ultraviolet light, R_F 0·11, 0·15 and 0·26; and a compound running at R_F 0·38 and giving a strong blue colour with the phosphate reagents.

The rest of the phenol eluate was run in a narrow band in propanol–aq. NH_3 soln.–water and, after drying the paper, the three ultraviolet-absorbing components were located. As separate elution of these three components resulted in three fractions each of which appeared still to contain all three components, further attempts to separate them were abandoned and they were eluted together, concentrated *in vacuo* and dissolved in water. The compounds were found to contain pentose and are presumably nucleotides.

The phosphate-containing compound running faster than the nucleotides in propanol–aq. NH_3 soln.–water was located by staining the edges of the paper as before, and was collected by elution and concentration. It was subsequently shown to be α-glycerophosphate (αGP).

The nucleotides accounted for about 113 μmoles of P/100 g. of calf lens and 146 μmoles of P/100 g. of rabbit lens, and the αGP for 97 μmoles of P/100 g. of calf lens and 146 μmoles of P/100 g. of young-rabbit lens.

It must be pointed out that the concentration of these nucleotides and of αGP are calculated from their relative proportions in the phenol eluate (R_F 0·18–0·50), since they did not show up with the molybdate reagent at an earlier stage. We were not able to elute all the phosphate from the paper, and it is therefore possible that either the αGP or the nucleotides were preferentially eluted, and concentrations given are not accurate. All the other compounds found in the lens were estimated before elution from paper.

A check on the concentrations of the glycerophosphate compounds in the lens is given by the following experiment. The alkali-stable, periodate-labile phosphate (Olley & Blewett, 1950) was measured in three batches of calf lens and found to be 381, 355 and 365 μmoles of P/100 g. of lens. The only alkali-stable, periodate-labile compounds we have found in the lens are αGP, GPC and GPE, and the figures agree well with the sum of the values found by chromatographic analysis for these three compounds (97 + 219 + 32 = 348 μmoles of P/100 g. of calf lens).

Nucleotides in acidic fraction from calf lens

This ultraviolet-absorbing fraction was chromatographed before and after mild or severe acid hydrolysis in solvents designed to separate nucleotides and purines and pyrimidine bases. Before hydrolysis, adenylic acid (AMP) and uridylic acid (UMP), both presumably in the 5′ form, seemed to be present, judged from the R_F in 2-methylpropan-2-ol–HCl–water and in propan-2-ol–HCl–water. There were no absorbing compounds without P. Eluted spots which corresponded in position to AMP or to UMP showed maximal absorption at 258 and 261 mμ in acid solution. Occasional traces of a compound with absorption maximum 250 mμ were found, suggesting the presence of a hypoxanthine nucleotide. After hydrolysis with N-HCl at 100° for 1 hr. adenine and UMP were obtained, judged by R_F values in 2-methylpropan-2-ol–HCl–water, P detection and absorption maxima of the eluted spots. After hydrolysis in 9N-$HClO_4$ at 100° for 1 hr., chromatography in the same solvent showed uracil and adenine. No ultraviolet-absorbing spot contained P after this treatment.

On one occasion the nucleotides prepared in this way liberated on hydrolysis not adenine but hypoxanthine.

Identification of α-glycerophosphate

The eluate contained a periodate-labile substance (Baddiley, Buchanan, Handschumacher & Prescott, 1956; Ansell & Norman, 1956), which was found to migrate at pH 4 in paper electrophoresis at the same rate as synthetic α-glycerophosphate. On treatment with acid phosphatase it was shown by paper electrophoresis that P_i and a substance giving a positive reaction with $AgNO_3$–NaOH (Trevelyan *et al.* 1950) was liberated. This substance had the same R_F value as glycerol, run simultaneously, in three solvents (propanol–aq. NH_3 soln.–water, phenol–water and butanol–acetic acid–water).

Spots of the eluate from lens and synthetic sodium α-glycerophosphate, chromatographed side by side in phenol–water, had different R_F values (0·35 and 0·19), whereas superimposed spots of the two compounds ran as one spot with an R_F value of 0·35. It was found that α-glycerophosphoric acid, neutralized with NaOH to pH 8, ran with R_F 0·19, whereas the same acid unneutralized, or neutralized with aq. NH_3 soln. to pH 8, had R_F 0·35. This accords with the fact that the lens extract, having been eluted from the Amberlite column IR (4B) OH with aq. NH_3 soln., would contain the ammonium salt of α-glycerophosphate, which has the higher R_F value.

It is possible that a similar explanation may be applied to the great variability in R_F values of certain phosphate compounds, including β-glycerophosphoric acid, reported by Olley & Dawson (1956), and also to the difference in R_F values between synthetic and naturally occurring phosphoglyceric acid (Threlfall, 1957).

Fraction precipitable by barium

This fraction was analysed chromatographically with *iso*butyric acid–aq. N-NH_3 soln. as solvent. This solvent separates the six nucleotides adenosine mono-, di- and tri-phosphate (AMP, ADP, ATP) and inosine mono-, di- and tri-phosphate (IMP, IDP, ITP) (Krebs & Hems, 1953). Four phosphorus-containing areas, all of which also absorbed in ultraviolet light, were found in both calf and rabbit lens, and these, together with P_i, accounted for nearly all the P in the fraction. The P_i ran together with ATP, and is easily located by means of the yellow colour it gives with the molybdate reagent. Since its concentration in the extract could be determined by a separate analysis, the value for P in ATP could be calculated by subtracting P_i from the total P found in the area. All the chromatographic analyses were done in duplicate and the agreement was good.

Two of the four phosphorus-containing areas corresponded in position on the chromatograms to ATP and ADP and were assumed to be these compounds without further identification. The presence of easily hydrolysable P in the lens has for a long time been taken as an indication of the presence of ATP and ADP. The fact that extracts of lens will convert glucose into lactic acid without the addition of ATP is proof of presence of this substance in the lens. Further proof comes from the measurement of the high-energy phosphate groups of ATP and ADP by enzymic methods (Pirie *et al.* 1955), and Dr Gustav Born, using an enzymic method specific for ATP (Strehler & Totter, 1954), has found ATP in calf and rabbit lens in amounts comparable with those found in the present work.

The two other components corresponded in position to IDP and ITP, but on the evidence given below it was concluded that they were uridine diphosphate (UDP) and uridine triphosphate (UTP) respectively. Table 5 gives an analysis of the fractions containing insoluble barium salts, obtained from the calf and rabbit lens. In the analysis reported in Table 5 the ultraviolet absorption of the UDP spot was weak in relation to the amount of P it contained and it seems probable that the UDP was mixed with compounds not absorbing in ultraviolet light. Hydrolysis of the extract containing insoluble barium salts in 9 N-$HClO_4$ for 1 hr. at 100° resulted in the formation of only two spots which absorbed in ultraviolet light, and which, when chromatographed, corresponded in position to uracil and adenine.

Characteristics of the nucleotides, probably uridine tri- and di-phosphate

The compounds were both shown to contain pentose. With the molybdate reagent they gave a blue–green colour, characteristic of easily hydrolysable phosphate compounds. The colour was not removed by subjecting the paper to an atmosphere of ammonia, again a characteristic of easily hydrolysable substances (Fleckenstein, Gerlach & Janke, 1953).

Spots of the chromatographed nucleotides, extracted in 0·01 N-HCl, both had a maximum absorption at 260 mμ. Incineration of similar spots showed that the 'UTP' had 3 atoms of P for each molecule of base. The actual values obtained for the number of atoms of P/molecule of uracil was 2·7, 2·7, 3·0 and 4·0. Incineration of the 'UDP' spot gave a value of 2·1 atoms of P/molecule of uracil and therefore, in this preparation, there did not appear to be appreciable amounts of other substances running with the 'UDP'.

The solutions of 'UTP' and 'UDP' were hydrolysed for 1 hr. in N-HCl at 100°. Measurement of the total P and inorganic P showed that approximately two-thirds and one-half respectively of the P in these compounds was acid-labile. Chromatography of the hydrolysates showed the liberation of P_i and the formation of a new substance containing P, absorbing in ultraviolet light and running in the position of uridylic acid (UMP) in propan-2-ol–HCl–water (Wyatt, 1951) and 2-methylpropan-2-ol–constant-boiling HCl–water (Smith & Markham, 1950).

Hydrolysis of the nucleotides in 9 N-$HClO_4$ for 1 hr. at 100° resulted in the formation of P_i and a

Table 5. *Analysis of fraction containing compounds having water-insoluble barium salts, in calf lens and young-rabbit lens*

Rabbits were about 3 months old. For preparation of the fraction see Methods section. The analysis was made chromatographically with *iso*butyric acid–aq. *N*-NH_3 soln. as solvent; for further details see text.

	P (μmoles/100 g. of lens)	
	Calf	Young rabbit
Trichloroacetic acid extract	1757	1660
Compounds having insoluble barium salts	870	844
ATP	347	448
ADP	60	46
UTP	128	88
UDP plus unknown	68	52
P_i	267	210

Table 6. *Trichloroacetic acid-insoluble phosphate in lens*

See text for preparation of extracts.

	P (μmoles/100 g. of lens)		
	TCA extract	Water suspension	TCA-insoluble
Calf	1620	2040	420
Rabbit	1650	2170	520

spot which absorbed in ultraviolet light but contained no P. Chromatography showed the substance to be the pyrimidine uracil. The evidence therefore supports the view that the nucleotides are UTP and UDP. In the absence of samples of the authentic compounds for comparison, however, it is not possible to establish this identity unequivocally.

Trichloroacetic acid-insoluble phosphate in lens

A lens was ground with five times its weight of water and a little sand in a pestle and mortar. To a known volume of the suspension an equal weight of 10% TCA (w/v) was added and the mixture was centrifuged. Total P was estimated in the lens suspension and the TCA extract. The results are shown in Table 6.

DISCUSSION

Tables 7 and 8 give the results of the analyses of the acid-soluble fractions of calf lens and young-rabbit lens respectively. In both about 85% of the acid-soluble phosphate is accounted for. Compounds whose presence in the lens extract is certain are PC, GPC, PE, GPE, αGP, ATP and ADP. Adenylic acid, uridylic acid and uridine di- and tri-phosphates are very probably present also, together with other nucleotides. Compounds which have been reported previously to be present at concentrations greater than 30 μmoles of P/100 g. of lens, but which have not been found by us, are creatine phosphate, hexose diphosphate, glucose 6-phosphate, glucose 1-phosphate, propanediolphosphate (Nordmann & Mandel, 1952) and fructose 6-phosphate (Frohman & Kinsey, 1952). Enzymic experiments had already shown the relative absence of all these compounds except propanediolphosphate (Pirie *et al.* 1955), and this compound has now been looked for by the method of Huggins & Miller (1956) but was not found. A comparison of the results of our analyses and those obtained by the method of LePage (1949) is given in Table 9.

The 15% of acid-soluble phosphate compounds not accounted for (220–260 μmoles of P/100 g. of lens) probably includes traces of the sugar phosphates mentioned above, phosphate compounds involved in the hexose monophosphate pathway, as well as other phosphate components of metabolic processes.

We do not know whether all the substances we have found are present in the living lens, or whether they are breakdown products formed in the fractionation processes. PC, GPC, PE and GPE have been found in many tissues and are not believed to be formed during the extraction process. The same applies to ATP, ADP and UTP. The other nucleotides, however, and the α-glycerophosphate, may or may not be formed from more complex molecules. We do not know of any report of the occurrence of αGP in tissues, determined by methods which differentiate between the free form and the choline and ethanolamine esters.

The fractionation process used here resulted in an appreciably higher proportion of orthophosphate (350 μmoles of P/100 g. of lens) than had been found in a trichloroacetic acid extract of lens, prepared quickly and in the cold. Nordmann & Mandel (1952) and Müller (1937) found 260–290 μmoles of orthophosphate/100 g. of calf lens and for the young-rabbit lens values of 171 μmoles of orthophosphate/100 g. of lens (Nordmann & Mandel, 1952) and 278 μmoles of orthophosphate/100 g. of lens (Palm, 1948) have been recorded. We do not know the source of the extra orthophosphate found in our experiments, but some of it probably comes from the breakdown of the pyridine nucleotides during the elution from the Amberlite IR (4B) OH column with aq. 3 N-ammonia solution.

The method of fractionation of lens extracts used was worked out for the analysis of the non-nucleotide portion of the acid-soluble phosphates. A more detailed analysis of the nucleotides in the fractions prepared in this way would not be profitable, since better methods are available for the analysis of nucleotides of tissue extracts (Hurlbert, Schmitz, Brumm & Potter, 1954).

The role of the five non-nucleotide phosphate compounds found in lens (αGP, PC, PE, GPC, GPE) is not known, and reactions in which they take part in other tissues have not been investigated in the lens. αGP has recently been shown to be involved in the formation of phosphatidic acids by tissue extracts (Kornberg & Pricer, 1953) and is an intermediary in the biosynthesis of phospholipids (Kennedy, 1953). It is formed in tissue extracts by the phosphorylation of glycerol with ATP, UTP or cytidine triphosphate (Bubitz & Kennedy, 1954). It is also formed as a side reaction of glycolysis by the reduction of dihydroxyacetone phosphate by the enzyme glycerophosphate dehydrogenase and reduced DPN.

PE and PC have been shown to be precursors of phosphatidylethanolamine and lecithin respectively (Dawson, 1954; Kennedy, 1956). They are probably both synthesized in tissues by phosphorylation of

Table 7. *Balance sheet of acid-soluble phosphorus compounds in the calf lens*

Analysis of the compounds with water-insoluble barium salts is given in Table 5.

	P (μmoles/100 g. of lens)					
Total	1757					
Insoluble barium salts	870		P_i	267		
		ATP, ADP, UTP		535		
					UDP + unknown	68
Soluble barium salts	887					
		'Basic'	371			
		'Acidic'	413			
					Unaccounted for	103
		Basic fraction	371			
			PC	81		
			GPC	219		
			PE	35		
			GPE	32		
					Unaccounted for	4
		Acidic fraction	413			
			PC	32		
			P_i	126		
			αGP	97		
		Nucleotides including AMP and UMP		113		
					Unaccounted for	45*
		Total accounted for		1537	Total unaccounted for	220

87% of the acid-soluble P compounds accounted for. Sum of P_i from the various fractions is 393 μmoles of P/100 g.
* R_F below 0·18 in phenol–water.

Table 8. *Balance sheet of acid-soluble phosphorus compounds in the young-rabbit lens*

Rabbits were 3 months old; average lens weight, 324 mg. Analysis of the compounds with water-insoluble barium salts is given in Table 5.

	P (μmoles/100 g. of lens)					
Total	1660					
Insoluble barium salts	844					
			P_i	209		
		ATP, ADP, UTP		582		
					UDP + unknown	53
Soluble barium salts	816					
		'Basic'	208			
		'Acidic'	608			
					Unaccounted for	—
		Basic fraction	208			
			PC	55		
			GPC	77		
			PE	13		
			GPE	29		
					Unaccounted for	34
		Acidic fraction	608			
			PC	—		
			P_i	143		
			αGP	146		
		Nucleotides including AMP and UMP		146		
					Unaccounted for	121*
						52†
		Total accounted for		1400	Total unaccounted for	260

84% of the acid-soluble P compounds accounted for. Sum of P_i from the various fractions is 355 μmoles of P/100 g.
* R_F below 0·2 in phenol–water.
† R_F above 0·2 in phenol–water and about 0·26 in propanol–aq. NH_3 soln.

Table 9. *Acid-soluble phosphates of lens. Comparison of the present findings with those obtained by the method of LePage* (1949)*

	P (μmoles/100 g. of lens)			
	Calf		Young rabbit	
	Present results	Method of LePage	Present results	Method of LePage
Total	1757	1765	1660	1534
P_i	393	231	355	171
Creatine phosphate	0†	127	0†	86
ATP + ADP	407	571	494	663
AMP	—	88	—	115
Nucleotides including AMP, UMP, UTP	241	—	234	—
Glucose 1-phosphate, glucose 6-phosphate, hexose diphosphate and phosphorylglyceric acid	12§	393	30†	402
Propanediol phosphate	0	135	0	Not done
Glycerophosphate	97	284‡	146	377‡
Glycerylphosphorylcholine	219	0	77	0
Glycerylphosphorylethanolamine	32	0	29	0
Phosphorylcholine	113	0	55	0
Phosphorylethanolamine	35	0	13	0

* Nordmann & Mandel (1952). † Pirie *et al.* (1955).
‡ Nordmann & Mandel (1952), by method of Fleury & Courtois (1941).
§ A. Pirie & R. van Heyningen (unpublished work).

the bases ethanolamine and choline in the presence of ATP (Ansell & Dawson, 1951; Wittenberg & Kornberg, 1953). GPE and GPC are not believed to be precursors of phospholipids, but may be breakdown products (Dawson, 1955*b*). Phospholipids in the cattle lens are found at a concentration of about 200 mg./100 g. of lens; most of these contain choline (Krause, 1935; D'Asaro, Young & Williams, 1954). This is equivalent to about 266 μmoles of P/100 g. of lens and is therefore the largest part of the TCA-insoluble phosphorus (Table 6).

Phosphorylcholine accounts for about one-tenth of the total acid-soluble phosphate of lens. It is present in the same proportion as it is in liver and the actual amount present in calf lens (av. 25 mg./100 g. of lens) is equal to that in rat spleen and intestine and only surpassed by the amounts in liver and testis (Dawson, 1955*a*). It is not possible to assess the importance of a substance in the metabolism of a tissue from a measure of its concentration, but it is possible that lipid metabolism in lens may be more important than we have thought hitherto and that glycerophosphate and phosphorylcholine may actively participate.

SUMMARY

1. About 85% of the phosphorus in the acid-soluble phosphate compounds of the lens of the calf and young rabbit have been accounted for.

2. The compounds which have been identified and estimated and which have not previously been found in the lens are phosphorylcholine, glycerylphosphorylcholine, phosphorylethanolamine and glycerylphosphorylethanolamine. Uridine 5′-phosphate, uridine diphosphate and uridine triphosphate are also probably present at fairly high concentrations.

3. In addition, the presence of α-glycerophosphate, adenosine 5′-phosphate, adenosine triphosphate and adenosine diphosphate has been confirmed.

4. The low concentration of hexose phosphate and phosphoglyceric acid in lens extracts is confirmed.

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The Reactivation of Cholinesterase Inhibited with Organophosphorus Compounds

1. REACTIVATION BY 2-OXOALDOXIMES

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The ability of many chemical compounds to restore the activity of cholinesterase (ChE) after inhibition by organophosphorus compounds is now well established (Childs, Davies, Green & Rutland, 1955; Wilson, 1955). It has also been shown that recovery of enzymic activity is due to chemical displacement of the phosphoryl group of the inhibitor from the group to which it is attached at or near the active centre of the enzyme (Davies & Green, 1956; Jandorf, Crowell & Levin, 1955). The most powerful reactivators, hydroxamic acids and oximes, will react rapidly with many organophosphorus inhibitors themselves (Hackley, Plapinger, Stolberg & Wagner-Jauregg, 1955; Green & Saville, 1956), but there is little correlation between these reactivities and the ability of hydroxamic acids and oximes to reactivate the inhibited enzyme (Childs *et al.* 1955). It has been suggested (Davies & Green, 1956) that this lack of correlation may be due to the formation of a preliminary complex between the reactivator and the inhibited enzyme. In this and the subsequent paper, this possibility has been tested kinetically for the two most potent groups of reactivating agents, 2-oxoaldoximes (I) and quaternized pyridine aldoximes.

(I)

$$R{\cdot}CO{\cdot}CH{:}N{\cdot}OH$$

(R = methyl, ethyl, *n*-propyl, *iso*propyl, *tert.*-butyl, phenyl and *cyclo*hexyl).

EXPERIMENTAL

Materials

The oximes were prepared by the action of nitrous acid on compounds possessing a suitably activated methylene group. *iso*Nitrosoacetophenone (I, R = phenyl) was obtained from acetophenone (Claisen & Manasse, 1887); the remaining compounds were obtained from β-oxo esters ($R{\cdot}CO{\cdot}CH_2{\cdot}CO_2{\cdot}Et$) by Freon's method (1939) for *iso*nitrosoacetone. *2-Oxo-n-valeraldoxime* (I, R = *n*-propyl), m.p. 49–50° (Found: N, 12·8. $C_5H_9O_2N$ requires N, 12·2%), *2-oxo-isovaleraldoxime* (I, R = *iso*propyl), m.p. 32–33° (Found: N, 12·2%), *2-oxo-2-cyclohexylacetaldoxime* (I, R = *cyclo*hexyl), m.p. 72–74° (Found: N, 9·2. $C_7H_{11}O_2N$ requires N, 9·0%) and *2-oxo-3:3-dimethylbutyraldoxime* (I, R = *tert.*-butyl), m.p. 52–53° (Found: N, 10·7. $C_6H_{11}O_2N$ requires N, 10·9%), have not previously been described.

Methods

Reactivation experiments. The ChE of human erythrocytes was inhibited by treatment with a dilute aqueous solution of tetraethylpyrophosphate (TEPP) or *iso*propyl methylphosphonofluoridate (Sarin). Saline suspensions of the inhibited cells were mixed with the oxime in barbitone buffer, pH 7·4, at 25°. At suitable time intervals samples were withdrawn and the ChE activity was measured by the electrometric method (Michel, 1949). The experimental procedure and the method of calculating the rate constants have been described fully (Davies & Green, 1956).

Reaction rates. During the reaction between 2-oxoaldoximes and Sarin, 3 moles of acid are liberated. The reaction rates were measured by continuous titration of this acid with alkali as described in detail elsewhere (Green & Saville, 1956).

Dissociation constants. These were given by the pH of an aqueous solution of the oxime half-neutralized with alkali.

RESULTS

Complex formation in reactivation by isonitrosoacetone (I, R = methyl)

If the formation of a complex between the reactivating agent and the inhibited enzyme is an essential preliminary step in reactivation, then this process may be represented by a set of equations similar to those already familiar in the Michaelis–Menten scheme for enzyme–substrate interaction,

$$EI + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI.A \xrightarrow{k_3} E + IA,$$

where EI is the inhibited enzyme, A the reactivator and EI.A the intermediate complex.

If the formation of the complex and its dissociation back into its components are rapid compared with its breakdown to give the free enzyme, then the overall rate of reactivation will be given by

$$v = dE/dt = k_3[EI.A] = k_3[EI_{total}][A]/[A] + K, \quad (1)$$

where $K = [EI][A]/[EI.A] = k_2/k_1$

and $[EI_{total}] = [EI] + [EI.A]$.

In each experiment the rate of reactivation by *iso*nitrosoacetone, which was in vast excess over the inhibited enzyme, was first-order with respect to the reactivator, i.e.

$$v = k_{observed}[EI_{total}]. \quad (2)$$

But, if complex-formation occurred, the rate would be given by equation (1) above, so that

$$k_{observed} = k_3[A]/K + [A], \quad (3)$$

which may be rearranged in the form

$$1/k_{observed} = K/k_3[A] + 1/k_3. \quad (4)$$

In Fig. 1, $1/k_{observed}$ is plotted against 1/[A] for the reactivation by *iso*nitrosoacetone of ChE inhibited with TEPP or with Sarin. A linear relationship, consistent with (4), is found in both cases. The values of K and k_3, obtained from the slopes and intercepts, are 0·02 mole/l. and 0·16 min.$^{-1}$ for ChE inhibited with TEPP, and 0·01 mole/l. and 0·24 min.$^{-1}$ for ChE inhibited with Sarin.

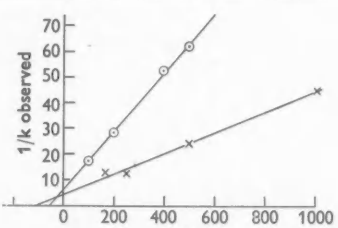


Fig. 1. Reactivation by *iso*nitrosoacetone of erythrocyte cholinesterase inhibited with TEPP (⊙) or Sarin (×). [A] is the concentration (mole/l.) of *iso*nitrosoacetone and k is the observed rate constant (min.$^{-1}$).

Reactivation by other 2-oxoaldoximes

A number of 2-oxoaldoximes (I) have been studied which have similar dissociation constants and reactivities with Sarin, but in which the alkyl group R was varied extensively. Table 1 shows the rates of reactivation by these compounds of ChE inhibited with TEPP or with Sarin. Preliminary experiments showed that, at the concentrations of reactivator selected, the observed rate of reactivation was roughly proportional to the concentration. This will only occur in dilute solution where $K \gg [A]$ and when (1) becomes

$$v = k_3[EI_{total}][A]/K \quad \text{and} \quad k_{observed} = k_3[A]/K.$$

DISCUSSION

The reactivities of hydroxamic acids and oximes with organophosphorus compounds are fairly closely related to the dissociation constants of the hydroxamic acids or oximes, although other factors do

Table 1. *Reactivation of erythrocyte cholinesterase inhibited with tetraethylpyrophosphate or with Sarin by 2-oxoaldoximes* (R·CO·CH:N·OH) *at* pH 7·4 *and* 25°

Concentration of the reactivator is 0·005 M for the TEPP-inhibited enzyme and 0·002 M for the Sarin-inhibited enzyme, except where otherwise stated. The rate constant given for the reaction between Sarin and the *tert.*-butyl compound is less certain than those for the other compounds, since with this particular compound the acid was not liberated at a first-order rate throughout the whole course of the experiment.

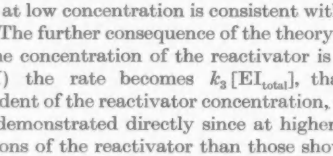
R	Second-order-rate constant (l./mole/min.) for reactivation of ChE inhibited with TEPP	Second-order-rate constant (l./mole/min.) for reactivation of ChE inhibited with Sarin	Second-order-rate constant (l./mole/min.) for reaction with Sarin at pH 7·4 and 25°	pK_a
CH_3	7	21	28	8·3
C_2H_5	11	20	24	8·4
n-C_3H_7	—	24*	28	8·3
iso-C_3H_7	—	24†	26	8·3
*cyclo*Hexyl	8·3‡	11	23	8·4
tert.-C_4H_9	2·4	5	(10)	8·6
Phenyl	11	6	18	8·3

* Rate at 0·0004 M. † Rate at 0·0013 M. ‡ Rate at 0·002 M.

have some influence, particularly with oximes (Green & Saville, 1956). The correlation is sufficient to enable the relative reactivity of any hydroxamic acid or oxime with a particular organophosphorus compound to be predicted roughly from the structure and dissociation constant of the hydroxamic acid or oxime. But, when the abilities of these compounds to reactivate inhibited ChE are compared, marked differences are found. For example, picolinhydroxamic acid and pyrimidine-2-hydroxamic acid are much more effective in reactivating ChE inhibited with TEPP than are other hydroxamic acids of comparable reactivity with organophosphorus compounds (Childs *et al.* 1955). These differences are not explicable on an electronic basis, but suggest rather that there is a difference in mechanism between reaction with simple organophosphates and reactivation. This difference could be the formation of a complex between the reactivator and the inhibited enzyme before fission of the phosphorus–enzyme bond.

Fig. 1 shows that reactivation by *iso*nitrosoacetone at low concentration is consistent with this theory. The further consequence of the theory, that when the concentration of the reactivator is large ($[A] \gg K$) the rate becomes $k_3[EI_{total}]$, that is, independent of the reactivator concentration, could not be demonstrated directly since at higher concentrations of the reactivator than those shown in Fig. 1 the rate of reactivation was too fast to measure with any precision.

Previously (Davies & Green, 1956) it has been shown that the rates of reaction of nucleophilic reagents with organophosphorus compounds (II),



when R′ and R″ were varied, were in the same ratio as the rates of reactivation by *iso*nitrosoacetone of the corresponding inhibited enzyme (III, where E is some group in the enzyme).

From this parallelism it was argued that reactivation by *iso*nitrosoacetone was a simple chemical displacement reaction and that no complex formation occurred. However, this argument is valid only if the strength or orientation of the complex formed between the reactivator and the phosphorylated enzyme depends on the alkyl groups associated with the phosphorus atom. If the site on the enzyme to which the reactivator is bound is not sterically affected by these alkyl groups then the complex may not be influenced by them, in which case the above parallelism would not disprove the existence of an intermediate complex.

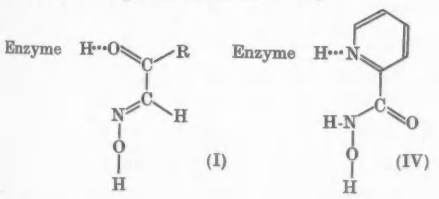
It is not obvious which part of the 2-oxoaldoxime molecule is responsible for the formation of the complex, but since these compounds do not possess a positive charge they are unlikely to be held by electrostatic attraction to the anionic site on the inhibited enzyme, which is believed to be shielded by the dialkylphosphoryl group (Wilson, 1955). The nucleophilic oximino group may contribute something to the binding force, although it will be shielded from direct contact with the enzyme surface by the phosphoryl group of the inhibitor and it is more likely that the reactivator is held by a hydrogen bond on to the carbonyl oxygen atom, or by van der Waals interaction between the enzyme and the alkyl group R.

The reactivation rates for the 2-oxoaldoximes given in Table 1 were measured under conditions where the observed rate constant ($k_{observed}$) is roughly k_3/K. Now k_3 measures the intrinsic ability of the oxime to displace the enzyme from the phosphoryl group so that, provided the orientation of the oximino group relative to the phosphoryl

group is not seriously changed by alteration in other parts of the oxime molecule, k_3 for a series of closely related oximes might be expected to run parallel to the intrinsic reactivity (as measured by the second-order-rate constant k') of these oximes with any other organophosphorus compound possessing the same dialkylphosphoryl group as the inhibited enzyme. For example, the values of k_3 for reactivation of the *iso*propyl methylphosphonyl enzyme by the oximes in Table 1 might be expected to be related to the rates of reaction of these oximes with Sarin. If this is true, any variations in K will be reflected by the ratio $k_{observed} : k'$.

Table 1 shows that variation of R from methyl to *iso*propyl has little effect on this ratio for ChE inhibited with Sarin, which suggests that the oxime is bound at the carbonyl group rather than by van der Waals interaction with the alkyl group. But, when the size of R is increased still further, to *cyclo*hexyl and *tert.*-butyl, the ratio $k_{observed} : k'$ begins to drop. When R is phenyl the relative ease of reactivation of ChE inhibited with TEPP or with Sarin becomes inverted. This would suggest that the steric requirement for hydrogen bonding on to the carbonyl oxygen atom is more stringent for the *iso*propyl methylphosphonyl enzyme than for the diethylphosphoryl enzyme.

Some indirect support for this concept is given by the variation in reactivating power of the isomeric pyridine hydroxamic acids. The most active, picolinhydroxamic acid (IV) (Childs *et al.* 1955; Jandorf *et al.* 1955; and Wilson & Ginsburg, 1955), possesses a nitrogen atom in a position analogous to that of the carbonyl oxygen atom in 2-oxoaldoximes (I) and could form complexes in the same way.



Nicotinhydroxamic acid and *iso*nicotinhydroxamic acid, in which the nitrogen atom is in the β- or γ-positions in the pyridine ring, could also be attached to the enzyme by hydrogen bonds to their heterocyclic nitrogen atoms, but these complexes would no longer have the nucleophilic oxygen atom in the hydroxyl group at the optimum position for reactivation to occur. Wilson, Ginsburg & Meislich (1955) have in fact shown that the dependence on concentration of the reactivation by nicotinhydroxamic acid of ChE inhibited with TEPP can be expressed by an equation of the form (1) consistent with the formation of such a complex. ChE inhibited with Sarin is reactivated by picolinhydroxamic acid (IV) much more slowly than is ChE inhibited with TEPP (Davies & Green, 1956), which is consistent with a reduced ability of the heterocyclic nitrogen atom to be held to the *iso*propyl methylphosphonyl enzyme by a hydrogen bond, owing to greater steric repulsion by the inhibited enzyme of adjacent parts of the pyridine ring in the hydroxamic acid.

SUMMARY

1. Kinetic studies on the reactivation by 2-oxoaldoximes (R·CO·CH:N·OH) of cholinesterase inhibited with organophosphorus compounds show that the formation of a complex between the reactivator and the inhibited enzyme is a likely step in the reactivation process.

2. Variation of the alkyl group (R) has only a minor effect on the reactivating power of 2-oxoaldoximes, which indicates that it is probably the carbonyl group in the oxoaldoxime which is linked to the enzyme in the complex.

3. An explanation is given for the marked differences in reactivating powers of the isomeric pyridine hydroxamic acids.

We are indebted to G. L. Sainsbury and M. Stansfield for the preparation of several of the oximes used in the above investigation. Acknowledgement is made to the Controller of H.M. Stationery Office for permission to publish this paper.

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The Reactivation of Cholinesterase Inhibited with Organophosphorus Compounds

2. REACTIVATION BY PYRIDINEALDOXIME METHIODIDES

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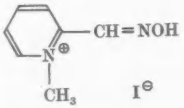
(*Received* 13 *May* 1957)

Pyridine-2-aldoxime methiodide (I) is by far the most effective compound yet found in restoring the activity of the enzyme cholinesterase (ChE) after inhibition with organophosphorus compounds (Davies & Green, 1955; Wilson & Ginsburg, 1955).



(I)

In the previous paper (Green & Smith, 1958) it was shown that the formation of complexes between the reactivating agent and the inhibited enzyme could assist reactivation very significantly. In the present paper we have examined the contribution of complex formation to the efficacy of pyridinealdoxime methiodides as reactivators of inhibited ChE.

EXPERIMENTAL

Materials

The pyridinealdoxime methiodides were obtained by boiling the oximes with methyl iodide in ethanol (Green & Saville, 1956). Quinoline-4-aldoxime methiodide, m.p. 232–233° (Found: I 40·2. $C_{11}H_{11}N_2OI$ requires I 40·4%) and *pyridine-2-aldoxime ethiodide*, m.p. 186–188° (Found: I 45·3. $C_8H_{11}N_2OI$ requires I 45·6%) were prepared similarly.

Reactivation experiments

These were carried out on human erythrocytes at 25° and pH 7·4 as described earlier (Davies & Green, 1956), whereby samples from a reactivation mixture containing inhibited enzyme and reactivator were taken at intervals and analysed for ChE activity by the electrometric method. With the higher concentrations of oxime, reactivation continued to an appreciable extent in the assay vessel despite the presence of excess (0·01 M) of acetylcholine, which gives the appearance of exceptionally rapid reactivation during the first few seconds of contact between the oxime and inhibited enzyme. For pyridine-2-aldoxime methiodide and ChE inhibited with tetraethylpyrophosphate (TEPP) or *iso*propyl methylphosphonofluoridate (Sarin) this effect is still noticeable even with a concentration of reactivator in the assay vessel of 10^{-5} M. The effect can be readily compensated for if the extent of the apparent rapid reactivation is taken as the enzyme activity at zero time of contact between inhibited enzyme and reactivator. However, since this reduces the difference between the measured enzyme activities at zero and infinite time, the percentage error is increased and the calculated rates are less accurate.

RESULTS

If complex formation is the first step in reactivation then the overall process may be described by the scheme:

$$\mathrm{EI} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EIA} \xrightarrow{k_3} \mathrm{E} + \mathrm{IA},$$

where EI is the inhibited enzyme, A the reactivator and EIA the complex.

Provided that the rates of formation of the complex and its dissociation into its components are large compared with the rate of breakdown of the complex to give free enzyme, the overall rate of reactivation is given by

$$v = k_3 [\mathrm{EI}_{\mathrm{total}}] [\mathrm{A}]/K + [\mathrm{A}], \quad (1)$$

where $K = [\mathrm{EI}] [\mathrm{A}]/[\mathrm{EIA}] = k_2/k_1$

and $[\mathrm{EI}_{\mathrm{total}}] = [\mathrm{EI}] + [\mathrm{EIA}]$.

If the reactivator is in large excess, as is always the case in these experiments, the observed velocity is given by

$$v = k_{\mathrm{observed}} [\mathrm{EI}_{\mathrm{total}}].$$

So that $$k_{\mathrm{observed}} = k_3 [\mathrm{A}]/K + [\mathrm{A}]. \quad (2)$$

In order to test the validity of this equation over a wide range of [A], it is best rearranged as originally proposed by Eadie (1942), namely

$$k_{\mathrm{observed}} = k_3 - k'K, \quad (3)$$

where k' is a parameter obtained by dividing k_{observed} by [A].

Fig. 1 shows the relationship between k_{observed} and the apparent second-order-rate constant k' for reactivation by pyridine-2-aldoxime methiodide and pyridine-4-aldoxime methiodide of ChE inhibited with TEPP. The agreement with equation (3) is within the experimental error. The values of k_3 and K obtained from the intercept and slope are given in Table 1.

Table 1. *Equilibrium constants K (moles/l.) and breakdown rates k_3 (min.$^{-1}$) of the intermediate complexes formed in the reactivation of erythrocyte cholinesterase*

Reactivator		ChE inhibited by: TEPP	Sarin	DFP	pK_a	Rate of† reaction with Sarin
Pyridine-2-aldoxime methiodide	K	0·00014	—	0·0008	8·0	33
	k_3	0·081	—	0·015		
Pyridine-4-aldoxime methiodide	K	0·0031	0·0008	—	8·5	47
	k_3	0·034	0·031	—		
*iso*Nitrosoacetone*	K	0·02	0·01	—	8·3	28
	k_3	0·16	0·24	—		

* K and k_3 taken from Green & Smith (1958).
† Rate constant (l./mole/min.) taken from Green & Saville (1956).

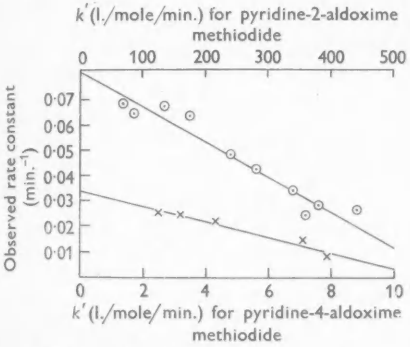


Fig. 1. Plot of the observed first-order-rate constant against the calculated second-order-rate constant (k') for reactivation of erythrocyte ChE inhibited with TEPP, by varied concentrations of pyridine-2-(⊙) and pyridine-4-(×)-aldoxime methiodides.

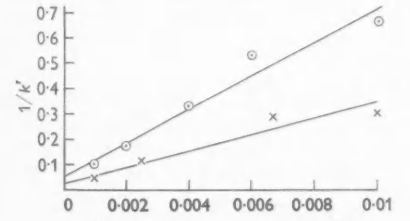


Fig. 2. Reactivation by pyridine-2-aldoxime methiodide (⊙) of erythrocyte ChE inhibited with DFP, and by pyridine-4-aldoxime methiodide (×) of erythrocyte ChE inhibited with Sarin. k' is the calculated second-order-rate constant (l./mole/min.).

With other inhibitors the experimental scatter was rather greater and it was impossible to prove adherence of the rate to equation (1) over a wide concentration range. Nevertheless, for ChE inhibited with either Sarin or di*iso*propyl phosphorofluoridate (DFP) the results were consistent with complex formation as shown below.

Equation (2) may be rearranged into the form

$$1/k' = K/k_3 + [A]/k_3. \quad (4)$$

In Fig. 2, $1/k'$ is plotted against [A] for reactivation by pyridine-2-aldoxime methiodide of ChE inhibited with DFP and by pyridine-4-aldoxime methiodide of ChE inhibited with Sarin. Both plots are consistent with equation (4). The values of k_3 and K obtained from the slopes and intercepts are included in Table 1; but, since reactivation in more dilute solution was too slow to enable satisfactory rate constants to be measured, these values of k_3 and K must be regarded as tentative.

To confirm the above values for k_3 and K some competitive studies were carried out with mixtures of reactivators. For a mixture of two reactivating agents, A and B, both of which form complexes, the observed first-order-rate constant is given by

$$k_{observed} = \frac{k_A K_B [A] + k_B K_A [B]}{K_A K_B + K_B [A] + K_A [B]}, \quad (5)$$

where k_A and k_B are the respective values of k_3, and K_A and K_B are those of K. Now, if [B] is kept constant and [A] is varied, then, provided that the values of K_A, K_B, [A] and [B] are such that K_B [A] is negligible compared with $K_A K_B + K_A$[B],

$$k_{observed} = \frac{k_A}{K_A}\left(\frac{K_B}{K_B + [B]}\right)[A] + \frac{k_B [B]}{K_B + [B]}. \quad (6)$$

In Fig. 3, the observed rate of reactivation is plotted against the concentration of *iso*nitrosoacetone (A) for the reactivation by mixtures of *iso*nitrosoacetone and pyridine-4-aldoxime methiodide of ChE inhibited with Sarin. From the slope, intercept and values of k_A and K_A for *iso*nitrosoacetone given in Table 1, $K_B = 0{\cdot}0012$ mole/l. and $k_B = 0{\cdot}037$ min.$^{-1}$, in fair agreement with the previous values.

In order to check equation (5) when K_B [A] is no longer negligible, mixtures of pyridine-2-aldoxime methiodide and *iso*nitrosoacetone were used to reactivate ChE inhibited with TEPP. The observed

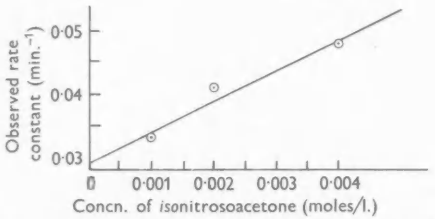


Fig. 3. Reactivation of erythrocyte ChE inhibited with Sarin by pyridine-4-aldoxime methiodide (0·005 M) plus varied concentrations of *iso*nitrosoacetone.

Table 2. *Rate of reactivation of erythrocyte cholinesterase inhibited with tetraethylpyrophosphate by mixtures of pyridine-2-aldoxime methiodide* (0·0001 M) *and varied concentrations of* isonitrosoacetone

Rate constants are in min.$^{-1}$.

Concn. of *iso*nitrosoacetone (M)	$k_{observed}$	
	Experimental	Calc. from equation (5)
0	0·0340	0·0338
0·001	0·0375	0·0372
0·002	0·0447	0·0408
0·004	0·0463	0·0470
0·005	0·0447	0·0498
0·007	0·0554	0·0552

rates are compared in Table 2 with those calculated from equation (5).

A few experiments have been carried out with other quaternized heterocyclic aldoximes. Pyridine-3-aldoxime methiodide was very much less active than either of its isomers, and at a concentration of 0·01 M would reactivate ChE inhibited with TEPP or with Sarin only slowly. Quinoline-4-aldoxime methiodide at 0·001 M was about as effective as pyridine-4-aldoxime methiodide against ChE inhibited with TEPP, and pyridine-2-aldoxime ethiodide showed activity comparable with that of the corresponding methiodide.

DISCUSSION

Kinetic data cannot in general be used to prove that a mechanism for a reaction is correct although they can show that a particular mechanism is incorrect. Normally they can be used only as confirmatory evidence for a mechanism which has been postulated on other grounds. The results given above do not show that the complex formation mechanism for reactivation is necessarily unique or correct, but they are consistent with this mechanism. It has also been necessary to assume that k_3 is much smaller than k_1 or k_2. Both the mechanism and the assumption about k_3 are reasonable since they are closely analogous to the situation in the more widely studied ChE–substrate action (see Bernhard, 1955). These reservations must be borne in mind in the following discussion.

The many hydroxylamine derivatives which have been found to reactivate inhibited ChE may be divided into two groups: those with positively-charged nitrogen atoms and those without. Wilson (1955) has pointed out a significant difference between these two groups, namely, that the members of the former group are about 20–100 times more effective in reactivating the diethylphosphoryl enzyme (e.g. ChE inhibited by TEPP) than in reactivating the di*iso*propylphosphoryl enzyme (e.g. ChE inhibited by DFP), whereas those in the latter group are only two to nine times more effective. This difference was attributed to 'promotion' of the intrinsic activity of the cationic compounds by interaction with the anionic site in the diethylphosphoryl enzyme.

The nature of this 'promotion' effect may be seen from equation (1). In dilute solution where $[A] \ll K$, (1) becomes

$$v = k_3 [EI] [A]/K. \quad (7)$$

If the strength of the complex is increased by electrostatic attraction between the cationic site in the reactivator and the anionic site in the inhibited enzyme, then the dissociation constant K will decrease, and the rate of reactivation v will correspondingly increase. The importance of this effect will depend on the distance apart of the two charged groups. This will depend on the extent to which the anionic site is sterically shielded by the dialkylphosphoryl group of the inhibitor. This shielding will be greater for the larger di*iso*propylphosphoryl group than for the diethylphosphoryl group, so that K will tend to be smaller for the diethylphosphoryl enzyme than for the di*iso*propylphosphoryl enzyme.

This effect is illustrated by the data for pyridine-2-aldoxime methiodide in Table 1. The intrinsic activity of the compound in detaching the dialkylphosphoryl group from the enzyme is measured by k_3, which is about five times as great for the diethylphosphoryl enzyme as for the di*iso*propylphosphoryl enzyme. This ratio is about the same as the ratio of the activities of non-cationic compounds (Wilson, 1955) which, in fact, is roughly the same as the ratio of the reactivities of nucleophilic reagents generally towards diethylphosphoryl and di*iso*propylphosphoryl compounds (Dostrovsky & Halmann, 1953). The 'promotion' effect is measured by the ratio of $1/K$ for the two phosphorylated enzymes. $1/K$ is about six times greater for the diethylphosphoryl enzyme than for the di*iso*propylphosphoryl enzyme, so that in dilute solution pyridine-2-aldoxime methiodide will reactivate the diethylphosphoryl enzyme about 30 times as fast as the di-

*iso*propylphosphoryl enzyme, as found by Wilson for other cationic compounds.

The combination of intrinsic activity and 'promotion' will account for many of the variations in activity observed in hydroxylamine derivatives. Thus nicotinhydroxamic acid methiodide, which shows the 'promotion' effect, is a superior reactivator of the diethylphosphoryl enzyme to nicotinhydroxamic acid (Wilson, Ginsburg & Meislich, 1955), although the latter will react about three times as rapidly with simple organophosphorus compounds at pH 7·4. Pyridine-2-aldoxime methiodide possesses both high reactivity and the ability to form strong complexes and is accordingly very effective as a reactivating agent.

The intrinsic activity of any reactivator will depend both on the ability of the reactivating agent to react directly with organophosphorus compounds and on the orientation and position of the reactivator in the complex. This latter factor may in some instances be critical and may account for the unexpectedly low reactivating power of pyridine-3-aldoxime methiodide.

The way in which the reactivator fits on to the enzyme surface will depend both on the geometry of the reactivator and inhibited enzyme and on the attraction between the cationic centre in the reactivator and the anionic site on the enzyme. Since the values of k_3 for reactivation of ChE inhibited with TEPP by pyridine-2- and -4-aldoxime methiodides differ by a factor of only about 2, and since the reactivities of these oximes with organophosphorus compounds are similar (see Table 1), it seems probable that the two oximes are orientated in a similar manner on the enzyme surface. If this is so, the quaternary nitrogen atom in the 4-isomer may be appreciably further from the anionic site on the enzyme surface than is the quaternary nitrogen atom in the 2-isomer, resulting in weaker binding and a much higher value for K as found experimentally.

Pyridine-2-aldoxime methiodide and *iso*nitrosoacetone are about equally reactive with organophosphorus compounds at pH 7·4, and it is of interest that the k_3 values also differ only by a factor of about 2, which is consistent with the complexes formed by both compounds being suitably orientated to facilitate reactivation. For ChE inhibited with Sarin, k_3 for pyridine-4-aldoxime methiodide is considerably less than that for *iso*nitrosoacetone, which would suggest that the pyridine compound is forced by the strong binding into a less satisfactory orientation for reactivation to occur.

SUMMARY

1. Kinetic studies of reactivation by pyridine-2- and -4-aldoxime methiodides of erythrocyte cholinesterase inhibited with organophosphorus compounds indicate that a preliminary step is the formation of a complex between the inhibited enzyme and the reactivator.

2. The reactivating powers of the isomeric pyridinealdoxime methiodides are compared in terms of their intrinsic reactivities with organophosphorus inhibitors, the strength of the complexes and the orientation of the reactivator in the complex.

Acknowledgement is made to the Controller of H.M. Stationery Office for permission to publish this paper.

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The *N*-Terminal Groups of Calf-Thymus Histones

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It is well established that the histones of calf-thymus nucleoprotein consist of a mixture of proteins, and partial fractionations of them have been achieved (e.g. Stedman & Stedman, 1951; Davison & Butler, 1954; Daly & Mirsky, 1955; Crampton, Moore & Stein, 1955). As a continuation of the work in this Laboratory, the study of the *N*-terminal groups of the mixed histones from calf-thymus glands was undertaken by the fluorodinitrobenzene method (Sanger, 1945). It was expected that the results would be helpful in controlling the fractionation of the histones. While this work was in progress a dissertation on this topic appeared (Haley, 1955) and the work described has since been

published as part of a wider study of histones (Luck *et al.* 1956). However, the results obtained here are not in good agreement with those reported.

MATERIALS AND METHODS

Preparation of histones. The mixed histones were prepared from previously isolated nucleoprotein from fresh frozen calf-thymus glands by the method of Davison, James, Shooter & Butler (1954). The series of preparations used were labelled H 1, H 16, H 17 and H 18. Some fractions of the histones were also examined. Thus the sodium chloride–ammonia treatment of the histones H 18 by the method of Davison & Shooter (1956) gave a histone precipitate (H 18 A) and soluble histones in the supernatant (H 18 B). The citric acid extraction of whole-thymus nucleoprotein (Davison & Butler, 1954) yielded a small amount of histone (labelled H 7), which was then separated into two fractions, H 7/1 and H 7/2, by column chromatography on carboxymethylcellulose by the method of Davison & Shooter (1956). In addition the whole of the proteins extracted by acid from thymus glands have been examined. For this purpose, thymus gland (50 g.) was blended in 0·25 N-HCl at room temperature, and the suspension centrifuged to remove large insoluble debris. The supernatant suspension was then dialysed until free of Cl^- ions and centrifuged in a Model L Spinco ultracentrifuge at 24 000 *g* (av.) for 1 hr. The clear supernatant was then freeze-dried (yield 2·0 g.) and examined for its *N*-terminal groups (preparation H 19).

Reaction with 1-*fluoro*-2:4-*dinitrobenzene.* A variety of conditions of coupling with 1-fluoro-2:4-dinitrobenzene (FDNB) have been tried, including an ethanol–bicarbonate medium (Sanger, 1945). In general, higher yields of total end groups were obtained in a guanidine hydrochloride–potassium bicarbonate medium, the purpose of which was to break up aggregates. A typical preparation is given below. The histone hydrochloride was dissolved in 6 M-guanidine hydrochloride to give a concentration of 10–20 mg./ml. Solid $KHCO_3$ was added to a concentration of 10–15 mg./ml., and FDNB to 0·05–0·1 ml./ml. The mixture was shaken at about 20° for 6–24 hr. It was then acidified, diluted with 3 vol. of water and extracted once with ether, the layers being separated by centrifuging. The yellow precipitate was washed repeatedly with N-HCl and centrifuged (this did not clarify the supernatant if 0·1 N-HCl or water was used). Final traces of reagent and dinitrophenol were removed by three acetone washes and an ether wash.

The weight of dinitrophenylhistones (DNP-histones) obtained was 110–120 % of the weight of histones taken. The DNP-histones were then hydrolysed, generally for 4·5 hr., in 11·1 N-HCl at 100° (Porter & Sanger, 1948), since DNP-proline was found to be an end group. The completeness of liberation of the more stable derivatives, such as DNP-valine, was checked by hydrolyses in 6 N-HCl at 110° or 120°. Hydrolyses were done in sealed tubes, with 1·5 ml. of acid/20 mg. of DNP-histones, this being a convenient weight for one complete analysis. The hydrolysates were diluted with water and extracted four times with ether in glass-stoppered test tubes. Each ether extract was washed with 0·1 N-HCl to remove any entrained acid-soluble materials such as ε-DNP-lysine. The ether used in this work was freed from peroxide by passing through a column of activated alumina (Dasler & Bauer, 1946).

The ether-soluble materials, after sublimation of excess of dinitrophenol (Mills, 1952), were transferred in three lots of acetone to a two-dimensional chromatogram on no. 7 Whatman filter paper buffered with 0·05 M-phthalate buffer (pH 6). This was then run in the dark for 30–40 hr. with 2-methylbutan-2-ol saturated with the phthalate buffer (Blackburn & Lowther, 1951), dried at room temperature and run in the other dimension overnight with 1·5 M-sodium phosphate buffer (pH 6) (Levy, 1954), 22 ml./chromatogram being used. The papers were dried at room temperature in the dark and the spots eluted in water and measured as described by Levy (1954) and Fraenkel-Conrat, Harris & Levy (1955). An equally satisfactory solvent was butan-2-ol saturated with the phthalate buffer, which required only 15–20 hr. to run. The phosphate was used as above for the second run (see Fig. 1). These alcoholic solvents were found to be more convenient than the 'toluene–ammonia' solvent advocated by Fraenkel-Conrat *et al.* (1955), because no time was necessary for equilibration and because the solvent did not deteriorate. The 'toluene–ammonia' solvent was also undesirable in a laboratory where ninhydrin–amino acid determinations were being carried out.

The corrections necessary for the quantitative measurement of the *N*-terminal groups have been estimated by (*a*) serial measurement of each end group in samples given different periods of hydrolysis and (*b*) recovery experiments on the free DNP-amino acids hydrolysed in the presence of

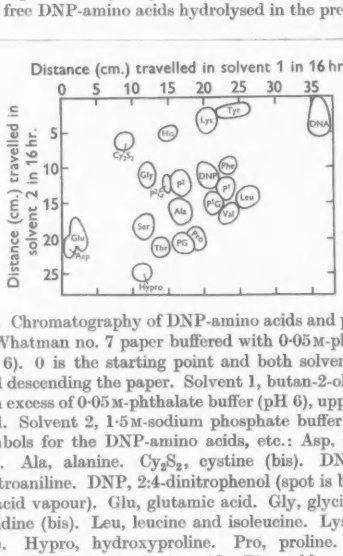

Fig. 1. Chromatography of DNP-amino acids and peptides on Whatman no. 7 paper buffered with 0·05 M-phthalate (pH 6). 0 is the starting point and both solvents were used descending the paper. Solvent 1, butan-2-ol shaken with excess of 0·05 M-phthalate buffer (pH 6), upper layer used. Solvent 2, 1·5 M-sodium phosphate buffer (pH 6). Symbols for the DNP-amino acids, etc.: Asp, aspartic acid. Ala, alanine. Cy_2S_2, cystine (bis). DNA, 2:4-dinitroaniline. DNP, 2:4-dinitrophenol (spot is bleached by acid vapour). Glu, glutamic acid. Gly, glycine, His, histidine (bis). Leu, leucine and isoleucine. Lys, lysine (bis). Hypro, hydroxyproline. Pro, proline. P^1, δ-chloro-α-DNP-aminovaleric acid. P^2, α-chloro-δ-DNP-aminovaleric acid. PG, prolylglycine [Hypro, Pro, PG and (more slowly) His, all darken in light]. P^1G, δ-chloro-α-DNP-aminovalerylglycine (DNP-methionine also runs here). P^2G, probably α-chloro-δ-DNP-aminovalerylglycine. Phe, phenylalanine. Ser, serine (ε-DNP-lysine also runs here). Thr, threonine. Tyr, tyrosine (bis). Val, valine. With 2-methylbutan-2-ol–phthalate buffer as solvent 1, the distribution of the derivatives is very similar, though the gap between the glutamic acid and serine is reduced.

unlabelled histones. In (*a*), in 11 N-HCl at 100°, the values for each end group rose to a maximum at 3–5 hr. hydrolysis and then fell. Extrapolation to zero time gave an initial value but required correction for the manipulative losses and was subject to the uncertainty that the rate of destruction of the DNP derivative in peptide form may have been different from the rate after its liberation. In (*b*), comparison of samples before and after hydrolysis indicated the manipulative losses in this method. These were 10–20%, depending on the derivative, but an average value of 20% loss has been adopted for all the derivatives encountered here. Much greater percentage losses occur where the amount of a given DNP-amino acid being handled is below 0·02 μmole.

The overall recoveries used in calculating the yields of end groups were: DNP-alanine 70%, bis-DNP-lysine 69%, DNP-valine and DNP-leucines 76%, DNP-aspartic and -glutamic acids 65%, DNP-serine 57%, DNP-glycine 41% and DNP-proline 33%. These all refer to 4·5 hr. hydrolysis at 100° in 11·1 N-HCl. The DNP-proline value was based on the recovery of small amounts of DNP-proline and relatively large amounts of its two degradation products, δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid (Scanes & Tozer, 1956). Trial degradations of DNP-prolylglycine (prepared from a sample of L-prolylglycine kindly given by Professor J. S. Fruton) and of DNP-proline in the presence of histones have been carried out. With DNP-proline the maximum yields of δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid were obtained after 0·75 and 1·5 hr. respectively in 11 N-HCl at 100°, whereas with DNP-prolylglycine the times were 3 and 5·5 hr. respectively, periods much closer to those observed in the hydrolysis of DNP-histones, in which the maximum yields of δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid were reached after 2·5 and 6 hr. respectively.

In 4·5 hr. hydrolysis the total recovery of DNP-proline and δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid from DNP-prolylglycine was 33%, and this value has been adopted as the value to be used with the DNP-histones. It is of interest that in the degradation of DNP-prolylglycine up to 2 or 3 hr. duration, spots due to δ-chloro-α-DNP-amino- and probably α-chloro-δ-DNP-amino-valerylglycine appear on the chromatograms as well as those of DNP-proline, 2:4-dinitrophenol and unchanged DNP-peptide. The positions of these substances as well as most of the common DNP-amino acids on the chromatograms are shown in Fig. 1.

The amount of DNP-proline itself on the chromatograms from DNP-histones is so small that it is unnecessary in the calculation of the yield of *N*-terminal proline to allow for the difference in molecular extinction coefficient between it and δ-chloro-α-DNP-amino- and α-chloro-δ-DNP-amino-valeric acid.

The acid-soluble part of the hydrolysate of DNP-histones was evaporated to dryness and run on Whatman no. 1 paper in butanol–acetic acid–water (5:1:4, by vol.). The ε-DNP-lysine formed a wide band which may also have contained α-DNP-histidine and DNP-arginine. After eluting the band with 0·1 N-HCl chromatographically the dried extract was coupled with FDNB (Levy, 1954). Extraction of the acidified products with ether removed all the ε-DNP-lysine as bis-DNP-lysine. No α-DNP-histidine or DNP-arginine could be detected on chromatograms of the acid-soluble remainder and no bis-DNP-histidine appeared either in the ether- or acid-soluble products.

The other part of the acid-soluble material, running more slowly than the ε-DNP-lysine band, was nearly colourless, though $N^{1(3)}$-DNP-histidine appeared as a grey band after exposure to light for some hours. All this slow-running material was eluted with 0·1 N-HCl, dried and recoupled with FDNB. This was done to estimate (as bis-DNP-lysine) the proportion of free lysine in the hydrolysates of the DNP-histones, since if the original reaction with histones was incomplete free lysine would appear in the hydrolysate (assuming that lysine ε-amino groups are not blocked by some steric effect or bonding). In three hydrolysates no bis-DNP-lysine appeared, whereas in a 6 N-HCl hydrolysate (24 hr. at 110°) of DNP-histones (H 1) a weak spot appeared amounting to less than 1% of the total lysine of the histones.

The nitroprusside reaction on acid hydrolysates of unlabelled histones and the Ehrlich test on chromatograms of barium hydroxide hydrolysates were both so weak that neither cystine nor tryptophan could contribute significantly to the end groups. The published analyses of histones show that these two acids are virtually absent (Davison & Butler, 1954; Crampton *et al.* 1955). The ether, acid and acetone washings of DNP-histones obtained during their preparation have been examined chromatographically. Only negligible traces of DNP-amino acids were detected.

The proportion of histones in the DNP-histones has been taken as 77%, being based on the number of DNP groups which could theoretically substitute in the whole histone according to the amino acid analysis published by Crampton *et al.* (1955).

RESULTS

The *N*-terminal groups found in the unfractionated histones were: proline and alanine, with smaller amounts of serine, lysine, leucine or isoleucine, valine, aspartic and glutamic acids, glycine and threonine. The proportions of the end groups (expressed as percentages of the total end groups) and the total quantities obtained in the guanidine–bicarbonate and ethanol–bicarbonate media are given in Table 1. By comparing the results from histones H 1 in each reaction medium, it can be seen from the weight of histone/mole of end group that the total yield is greater in the material prepared in guanidine–bicarbonate. The duration of coupling beyond about 6 hr. had little effect on the yield of end groups.

The values for serine and lysine end groups were found to vary considerably. It is thought that some of the earlier high values for serine were due to the omission of the acid washing of the ether-soluble end groups, which would result in traces of ε-DNP-lysine being measured as DNP-serine, since it runs in exactly the same position on the two-dimensional chromatograms. Nevertheless, the average yield (moles/g. of histone) of serine end groups from histone preparation H 1 was nearly four times as high when guanidine was present in the coupling medium (see Table 1), so that it would appear that in this case the ethanol–bicarbonate medium fails to allow 1-fluoro-2:4-dinitrobenzene to react completely with serine end groups. However, in the

later preparations of histones such as H 16 and H 17, even with the guanidine medium, the serine end groups have been as low as 5 % and the lysine values down to 1 % of the total.

An examination was also made of the fractions of the histones prepared by (*a*) extraction of the original nucleoprotein with citric acid (Davison & Butler, 1954) and (*b*) precipitation of the histones by sodium chloride–ammonia (Davison & Shooter, 1956). H 7/1 and H 7/2 are subfractions of the histones extracted by citric acid, which were obtained by chromatography on carboxymethylcellulose by the method of Davison & Shooter (1956). H 18 A and H 18 B are the sodium chloride–ammonia precipitate and supernatant products respectively derived from H 18, a mixture of histone preparations H 16 and H 17. The results are given in Table 2, together with those obtained from the proteins extracted from a thymus-gland brei by 0·25 N-HCl (sample H 19). This material includes much protein which is not histone. It can be seen that these various fractions gave results which differed markedly from the original histones described above.

Fractions H 7/1 and H 7/2 account for most of the histones extracted by citric acid (which are only approximately 2 % of the total histones in the nucleoprotein), and these fractions also account for most of the valine end groups found in the whole unfractionated histones. In another preparation of the citric acid-extracted histones, the proportions of the end groups in a 6 N-HCl hydrolysate (12 hr. at 110°) were: valine 39 %, alanine 19 %, lysine 15 %, others 27 %. DNP-proline and its products were completely destroyed under these conditions.

Three samples of DNP-histones (H 1) have been hydrolysed under more vigorous conditions to liberate any valine which might be present as DNP-valyl-peptides (such as the DNP-valyl-ε-DNP-lysine reported by Luck *et al.* 1956). Some valine peptides are notoriously stable to acid hydrolysis (Synge, 1945; Porter & Sanger, 1948). However, hydrolysis in 6 N-HCl at 108° for 8 hr., at 110° for 48 hr. or at 120° for 24 hr., though increasing the proportion of valine and leucine (or isoleucine) end groups, only raised them from about 2 to 5 or 6 % of the total end groups. No sign of the peptide DNP-valyl-ε-DNP-lysine was seen on any of these chromatograms, and only after very short hydrolyses, e.g. 1 hr. at 100° in 11 N-HCl, did any spots other than those in the positions of the known DNP-amino acids appear. Stable peptides were also

Table 1. *N-Terminal groups of unfractionated histones*

The proportions of *N*-terminal groups are expressed as percentages of the total end groups found.

	In guanidine hydrochloride–bicarbonate medium*			In ethanol–bicarbonate medium†
Histone preparation	H1	H16	H17	H1
N-Terminal amino acid				
Proline	33	45	48	40
Alanine	26	40	36	29
Serine	20	5	5	6
Lysine	7	1	2	11
Others‡	14	9	9	14
Wt. of histone/1 mole of end groups	22 500	29 000	29 000	28 000

* See Methods section.
† Ethanol 0·65 ml./ml., potassium bicarbonate 9 mg./ml., FDNB 0·03 ml./ml.
‡ Leucine (or isoleucine), valine, aspartic and glutamic acids, glycine and threonine.

Table 2. *N-Terminal groups of various histone fractions*

The proportions of the *N*-terminal groups are expressed as percentages of the total end groups found. All were coupled with FDNB in the guanidine–bicarbonate medium.

Histone fraction (for origin, see text) ...	H 7/1	H 7/2	H 18/A	H 18/B	H 19
N-Terminal amino acid					
Proline	0	39	16	66	35
Alanine	21	27	73	8	27
Serine	7	4	3·5	4	1
Lysine	16	6	2	10	3
Leucine(s)	9	5	Trace	Trace	6*
Valine	40	5	Trace	Trace	6*
Others	7	14	5·5	12	22
Wt. of histone/1 mole of end groups	62 000	14 000	27 000	27 000	45 000

* From samples hydrolysed in 6 N-HCl for 22 hr. at 110°.

sought in the ethyl acetate extracts of the acid-soluble part of many of the hydrolysates of DNP-histones, but without success. Under the usual conditions of hydrolysis (4·5 hr. at 100° in 11 N-HCl) only one spot appeared on chromatograms of such extracts, in the same position as DNP-serine. However, this spot was ninhydrin-positive and proved to be ε-DNP-lysine.

DISCUSSION

The *N*-terminal groups of the thymus histones found above indicate the presence of two major types of peptide chain and about eight other types present in minor or trace amounts. The average chain weight is about 25 000. This value and the nature of the chief end groups are, with the exception of alanine, at variance with the results of Haley (1955) and Luck *et al.* (1956), which were obtained with histones prepared by extraction with sodium chloride and hydrochloric acid of thymus-cell nuclei. In whole-thymus histones these authors found that valine accounted for 53 % of the *N*-terminal residues, leucine for 21 %, alanine for 18 % and glycine for 8 %. The value of 1 g. mol. of end groups/8000 g. of histones was supported by their sedimentation and osmotic-pressure studies.

It is difficult to account for these differences except on the assumption that degradation of the histones occurred in the preparative method used by Luck *et al.* (1956). Although the histones are known to aggregate in alkaline solution (Davison *et al.* 1954), the reaction with 1-fluoro-2:4-dinitrobenzene is thought to have been complete since virtually no free lysine could be detected in the hydrolysates of DNP-histones. The hydrolyses are thought to have liberated most of the end groups since more severe conditions did not raise the values of terminal DNP-valine and DNP-leucine (which can give very stable DNP-peptides) to values anywhere near those of DNP-proline or DNP-alanine. The corrections for hydrolytic and manipulative losses are thought to have been adequate, though in some work with DNP-proteins, e.g. with hypertensin (Elliott & Peart, 1957) and with corticotrophin (Levy, Geschwind & Li, 1955), the losses of terminal DNP-amino acids were greater than expected. Finally, the washings from the preparations of the DNP-histones have revealed no loss of terminal groups such as Thompson (1953) encountered in the study of carboxypeptidase.

Experiments made with the object of causing slight degradation of the histones, by keeping a solution at pH 7 for 2 days or in 0·1 N-sodium hydroxide for a day, or by heating the aqueous solution at 100° for an hour, caused only very slight increases in some of the end groups. Another experiment wherein all the acid-soluble protein (not merely the histones) from thymus glands was coupled, after dialysis, with 1-fluoro-2:4-dinitrobenzene showed that the amounts of valine and leucine end groups (each was 6 % of the total) were small compared with the proline and alanine end groups, which were 35 and 27 % of the total end groups respectively.

The sodium chloride–ammonia fractionation of the histones has been shown to precipitate the histones which are relatively richer in arginine and migrate faster on electrophoresis, and to leave in the solution the histones which are relatively richer in lysine and which migrate more slowly on electrophoresis (Davison & Shooter, 1956). Table 2 shows further that most of the arginine-rich histones (H 18 A) have alanine *N*-terminal groups and most of the lysine-rich ones (H 18 B) have proline *N*-terminal groups. The small amounts of histones with other end groups fall into both of these main classes.

The origin of the differences in yield of serine and lysine end groups in different preparations of the mixed histones has not been established. In the later preparations both of these acids formed only a minor proportion of the *N*-terminal groups. There may be variations in the proportions of the different histones obtained from different animals or breeds of cattle. Allfrey, Mirsky & Osawa (1955) have noted the variability in properties of thymus nuclei prepared at different times of the year. Moreover, in the preparative stages involving washings and dialyses near neutrality there is the possibility of enzyme action causing slight degradation of peptide chains.

SUMMARY

1. The chief *N*-terminal groups of the histones extracted from calf-thymus glands are proline and alanine, with smaller amounts of lysine and serine, and still less of six other amino acids.

2. The proportions of the serine and lysine end groups have declined markedly in later preparations of the histones.

3. The quantities of the end groups gave an average weight of about 25 000/g. mol. of end groups.

4. In a fractionation of the histones, those precipitated by sodium chloride–ammonia (the arginine-rich histones) mostly have alanine *N*-terminal groups, whereas those remaining in solution in sodium chloride–ammonia (the lysine-rich histones) mostly have proline end groups.

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Studies on the Biosynthesis of Riboflavin

4. PURINE METABOLISM AND RIBOFLAVIN SYNTHESIS IN *EREMOTHECIUM ASHBYII**

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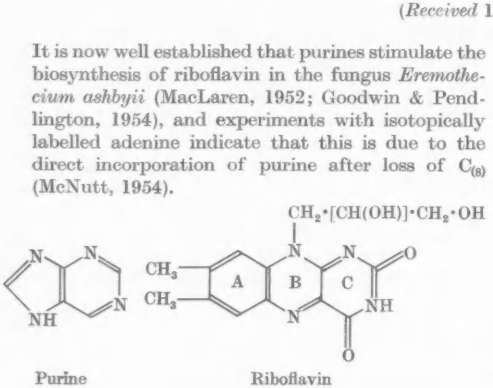
It is now well established that purines stimulate the biosynthesis of riboflavin in the fungus *Eremothecium ashbyii* (MacLaren, 1952; Goodwin & Pendlington, 1954), and experiments with isotopically labelled adenine indicate that this is due to the direct incorporation of purine after loss of $C_{(8)}$ (McNutt, 1954).



Purine Riboflavin

Experiments with labelled glycine, formate and serine also indicate that rings B and C of riboflavin are built up in a very similar pattern to the purine rings (Plaut, 1953; 1954*a*, *b*; Goodwin & Jones, 1956). Naturally occurring pyrimidines do not stimulate flavinogenesis (Brown, Goodwin & Pendlington, 1955).

Goodwin & Pendlington (1954) found that xanthine and adenine both stimulated flavinogenesis and that xanthine was the more efficient. As xanthine is structurally the purine most closely related to riboflavin, it is likely that other purines are active by virtue of their conversion into xanthine. One would expect that the first step in the conversion of adenine would be the formation of hypoxanthine, for an adenase (adenine deaminase) has been observed in a number of micro-organisms, especially fungi (Di Carlo, Schultz & McManus, 1951; Roush, 1954). The hypoxanthine produced would then be converted into xanthine by xanthine oxidase. Preliminary experiments showed, however, that hypoxanthine is much less active than adenine and thus would appear to be ruled out as an intermediate.

Because of these somewhat unexpected results it was decided (*a*) to extend the number of purines investigated to include hypoxanthine, uric acid and guanine, the ribosides adenosine and inosine, and the ribotide adenosine-3′-phosphate; (*b*) to follow the metabolism of these compounds during their incorporation into riboflavin, and (*c*) to examine the effect on riboflavin synthesis of certain purine antagonists and inhibitors of purine synthesis.

A brief report of some of these findings has already appeared (Brown, Goodwin & Jones, 1956).

* Part 3: Goodwin & Jones (1956).

EXPERIMENTAL

Cultures. The strongly flavinogenic strain of *Eremothecium ashbyii* used throughout the investigation was kindly supplied by Mrs J. Robson (Atomic Energy Research Establishment, Harwell). Apart from yielding more riboflavin it behaved towards purines in the same way as our original strain (Goodwin & Pendlington, 1954).

Media. The basal medium was that described previously (Goodwin & Pendlington, 1954). Appropriate amounts of bacteriological peptone (Evans) and the compounds under test were added to the medium, which was adjusted to pH 5·8 and generally sterilized by autoclaving at 15 lb./in.^{2}. When the compounds in use were known to be unstable to autoclaving, the media were sterilized by filtration.

Materials. The naturally occurring purines, their ribosides and ribotides were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. They were checked for purity by paper chromatography. The azapurines were a gift from Dr R. E. F. Matthews (Molteno Institute, Cambridge); azaserine and the triazoles were a gift from I.C.I. Pharmaceuticals Ltd. (Dr A. W. Crowther); the substituted pyrimidines and 6-mercaptopurine were a gift from the Wellcome Foundation (Dr L. G. Goodwin); the antibiotics were a gift from The Lederle Laboratories (Dr F. C. Ottati) and riboflavin and *iso*riboflavin were a gift from Roche Products Ltd. (Dr A. L. Morrison).

Cultural conditions. These have been previously described in detail (Goodwin & Pendlington, 1954).

Determination of dry weight and riboflavin. The methods employed have been described (Goodwin & Pendlington, 1954). Analyses were carried out in triplicate. The reproducibility of results with *E. ashbyii* was given in detail by Goodwin & Pendlington (1954) and Brown *et al.* (1955), and in order to conserve space the scatter of the results will not be given in the tables to follow.

Paper chromatography. The media to be examined were spotted directly on to Whatman no. 1 paper and chromatographed in a descending system, either propan-2-ol (170 ml.) –conc. HCl (sp.gr. 1·19; 41 ml.)–water (to 250 ml.) or butan-1-ol–acetic acid–water (4:1:5, by vol.) being used as solvent system. The former is recommended for separation of purines, nucleosides and nucleotides (Wyatt, 1951) and the latter for riboflavin and its derivatives (Crammer, 1948). It was found that both solvent systems gave good separations of both groups of compounds; they could therefore be used for checking the identity of the various components of the media. The chromatograms were developed for about 20 hr. in a tank darkened to prevent photolysis of riboflavin and its derivatives. After removal from the tanks the sheets were dried in the dark in a current of warm air for 15 min. The purines and their ribosides or ribotides were located with ultraviolet light from a low-pressure Hg lamp emitting mainly the Hg line at 256 mμ. The compounds show up as dark spots on a blue–white background (Wyatt, 1955). Riboflavin and its derivatives were also visualized by ultraviolet light, but in this case a high-pressure Hg lamp (Hanovia) emitting mainly the Hg line at 365 mμ was used. Under this lamp the flavins fluoresce bright yellow or bright blue.

Elution of the purine spots was carried out with 0·1 N-HCl by the method of Dent (1947); the flavin derivatives were similarly eluted with water. These eluted spots were then examined spectrophotometrically, the eluate from a blank piece of chromatography paper of the same area and R_F as the spot under consideration being used in the compensating cell.

Spectrophotometric determination of adenine and hypoxanthine in the presence of riboflavin

Kalckar (1947) showed that in a system in which adenine is being converted into hypoxanthine without any net change in total purine, the amount of hypoxanthine formed can be calculated from measurements of the changes in extinction (ΔE) at 265 and 240 mμ. The production of 1 μg. (0·0074 μmole) of hypoxanthine/ml. of solution is indicated by $\Delta E_{265\ m\mu}$ (1 cm.) −0·059 and $\Delta E_{240\ m\mu}$ (1 cm.) +0·032. This calculation cannot be directly applied when, as in the metabolism of adenine by *E. ashbyii*, (*a*) interference in the region 240–265 mμ is caused by another substance, and (*b*) the total purine concentration is falling. In the present case, chromatographic analysis and absorption-spectral measurements of media not containing purines showed that riboflavin was the only interfering substance. Allowance for

Table 1. *Spectrophotometric determination of adenine and hypoxanthine in the presence of riboflavin*

Initial adenine concn. 0·875 mM (≡0·875 μmole/ml.). See Experimental section for details of calculation.

Medium	10^{-1} E values: 240 mμ	251 mμ	265 mμ	445 mμ
1. Uninoculated	0·560	0·985	1·105	—
2. Culture (5 days)	0·642	0·984	0·981	0·0260
3. Culture (5 days), corrected for riboflavin	0·619	0·945	0·914	—
4. Values calc. for (3) assuming only adenine present	0·536	0·945	1·062	—
ΔE	0·083	—	−0·148	—

Adenine metabolized to compounds other than hypoxanthine = [(0·985–0·945)/0·985] × 100 = 4·1 %.
Amount of hypoxanthine produced (μmole/ml.) =

(*a*) (0·083 × 10 × 0·0074)/0·032* = 0·191
(*b*) (−0·148 × 10 × 0·0074)/−0·059* = 0·186
} 0·188.

Adenine converted into hypoxanthine = (0·188/0·875) × 100 = 21·5 %.
Therefore adenine unchanged = 74·4 %.

* Values given by Kalckar (1947).

riboflavin absorption in the 240–265 mμ region can be made by measuring $E_{445\,m\mu}$ (the maximum of riboflavin absorption in the visible region of the spectrum) and from the absorption spectrum of pure riboflavin, calculating its contribution to the observed $E_{240-265\,m\mu}$ values.

As hypoxanthine and adenine exhibit an isosbestic point at 251 mμ at the pH of the medium (5·8), any difference between $E_{251\,m\mu}$ corrected for riboflavin absorption and $E_{251\,m\mu}$ of the uninoculated medium represents the amount of adenine which has been metabolized but *not* to hypoxanthine. The $E_{240\,m\mu}$ and $E_{265\,m\mu}$ values corresponding to $E_{251\,m\mu}$ (corrected), if all the purine remaining were adenine, can be calculated, and consequently the amount of hypoxanthine (μg./ml.) formed is given by $E_{265\,m\mu}$ (corr.) $- E_{265\,m\mu}$ (calc.)/ $-0{\cdot}059$, or $E_{240\,m\mu}$ (corr.) $- E_{240\,m\mu}$ (calc.)/0·032. An example (Table 1) will illustrate the steps in the calculation. A similar calculation can be made for the adenosine–inosine system. After making allowances for the various corrections to be applied, the method is probably accurate to ±5%.

Spectrophotometric measurements were carried out with the Unicam SP. 500 and SP. 600 instruments.

RESULTS

Relative efficiency of purines and purine derivatives. Previously the flavinogenic activity of only xanthine and adenine had been reported (Goodwin & Pendlington, 1954). These purines have now been compared with hypoxanthine, guanine and uric acid. Adenosine has been re-examined because the observation of McNutt (1954), that adenosine is less effective than adenine, was not in agreement with our previous results (Goodwin & Pendlington, 1954). Adenosine-3′-phosphate and inosine were also tested.

Table 2 gives the results of a typical series of experiments. The important points in regard to flavinogenic activity are (*a*) guanine is better than xanthine, (*b*) hypoxanthine, inosine and uric acid are much poorer than adenine, and (*c*) adenosine and adenosine 3′-phosphate are very similar to adenine. Furthermore, none of the compounds tested stimulates growth. The weak action of hypoxanthine and uric acid tend to support the results of MacLaren (1952), but the observations with guanine, which were repeated many times, are unexpected but confirm a single observation of McNutt (1954). As *E. ashbyii* stores riboflavin in its mycelium as well as excreting it into the medium, a possible explanation of the quantitative differences between the purines was the differential accumulation of riboflavin in the mycelium in the presence of certain members. Table 3 shows that the riboflavin present in the mycelium remains constant over the

Table 2. *Effect of various purines, nucleosides and nucleotides on flavinogenesis by* E. ashbyii

Basal medium +4 mg. of peptone N/100 ml. and 0·37 mM-purine.* 5-day cultures, static; temp. 28°; triplicate determinations: amounts were produced in 15 ml. of medium in 50 ml. conical flasks.

Purine added	Mycelium dry wt. (mg.)	Riboflavin	
		Total (μg.)	Amount (g./100 g. of dry mycelium)
None (control)	12·0	583	4·86
Adenine	12·5	920	7·35
Hypoxanthine	12·5	742	5·93
Xanthine	11·8	930	7·87
Guanine	12·2	1010	8·27
Uric acid	12·8	715	5·58
Adenosine	13·0	907	6·97
Inosine	12·9	723	5·32
Adenosine-3′-phosphate	13·0	870	6·69

* This value was chosen because it represents about the maximal solubility of guanine in the basal medium.

Table 3. *Riboflavin distribution in mycelium and medium in cultures of* E. ashbyii *grown in the presence of various purines, nucleosides and nucleotides*

Experimental details were as given in Table 2.

Purine added	Riboflavin (μg.)			
	In medium	In mycelium	Total	% of total in mycelium
None (control)	340	104	444	23
Adenine	575	147	722	20
Hypoxanthine	458	147	605	24
Xanthine	578	146	724	20
Guanine	652	139	791	18
Uric acid	450	130	580	22
Adenosine	572	135	707	19
Inosine	451	124	575	22
Adenosine-3′-phosphate	533	122	655	19

range 18–24% of the total, irrespective of the purine present. This confirms the observations of Goodwin & Pendlington (1954) made with other media, that the percentage of riboflavin in the mycelium is reasonably constant, although with their original strain of *E. ashbyii* it tended to be somewhat higher than that observed with the present strain. Dry weights could not be recorded in this experiment because the mycelia are destroyed by hydrolysis with N-HCl to liberate the bound riboflavin. Table 3 also confirms the results given in Table 2 concerning the relative efficiencies of the various purines.

Metabolism of adenine and adenosine by E. ashybii

Cultures of *E. ashbyii* metabolizing adenine and adenosine were examined chromatographically on paper and, apart from the original compounds, spots corresponding to hypoxanthine and inosine respectively were detected. The identity of these two compounds was confirmed by mixed chromatography with authentic specimens in two solvent systems (see Experimental) and by their absorption spectra after elution from the paper. Apart from large amounts of riboflavin, traces of lumichrome and flavinadenine dinucleotide, no other fluorescing or ultraviolet light-absorbing spots were detected. Specially significant was the absence of any trace of xanthine or xanthosine.

The spectrophotometric method for determining adenine and hypoxanthine in the presence of riboflavin was applied in a series of experiments involving media containing varying amounts of adenine. The results of a typical experiment given in Table 4 show that the percentage of 'adenine metabolized to compounds other than hypoxanthine' (ANH) is high at low initial concentrations and gradually decreases with increasing adenine concentrations; simultaneously, with the exception of a drop at 0·3 mM, the percentage of adenine not attacked also increases with increasing adenine concentrations. The percentage of adenine converted into hypoxanthine reaches a maximum at an adenine level of about 0·3 mM. This tendency, in association with a low residual adenine level, was observed in all similar experiments but the actual adenine level at which the peak was observed varied from 0·3 to 1 mM. When the amounts of adenine metabolized (Fig. 1) are plotted it is clear that (*a*) the amount of hypoxanthine formed and the ANH fraction both tend to become constant in the region 0·3–0·6 mM-adenine, (*b*) the amount of unchanged adenine increases regularly with increasing adenine concentration, (*c*) the curve for riboflavin production tends to follow the curves for hypoxanthine and ANH, but above 0·6 mM-adenine it corresponds more closely to the ANH curve. The correspondence between the riboflavin and ANH curves is much more marked with adenosine (see later). It is specially interesting that riboflavin stimulation and ANH both tend to level out in the region 0·3 mM-adenine. This corresponds to maximal relative formation of hypoxanthine. In retrospect, this tendency for riboflavin stimulation to slow down in the region corresponding to 0·3 mM-adenine can be seen in our original results (Goodwin & Pendlington, 1954).

The efficiency of the conversion of adenine into riboflavin, as measured by the percentage of the

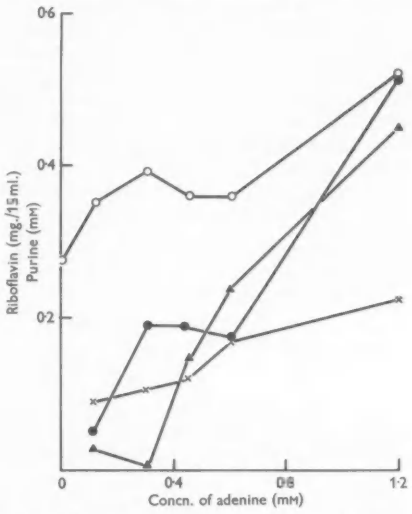


Fig. 1. Effect of concentration on the metabolism of adenine by *E. ashbyii*. Experimental conditions were as described in Table 2. ○, Riboflavin in medium; ×, ANH; ●, hypoxanthine produced; ▲, residual adenine.

Table 4. *Influence of initial adenine concentration on adenine metabolism by* E. ashbyii

Experimental conditions were as given in Table 2.

	Percentage converted				
Initial concn. of adenine (mM) ...	0·15	0·30	0·45	0·60	1·2
Conversion into hypoxanthine	28	64	43	31	43
ANH	57	34	28	29	19
Unchanged	15	2	29	40	38

ANH fraction which can be accounted for as riboflavin, is given in Table 5. On the assumption that the ANH fraction is incorporated into all the riboflavin produced (McNutt, 1954), the efficiency drops from essentially 100% at the lowest adenine level to 55% at the 0·6 mM level; the apparent increased efficiency at the 1·2 mM level reflects the increase in riboflavin synthesis after the plateau region is passed. If ANH is assumed to contribute only to that riboflavin produced above the basal level, then apart from one anomalous result at the lowest adenine concentration, the incorporation varies between 11 and 37% over the concentration range studied.

When the metabolism of adenosine is compared with that of adenine on an equimolar basis over a range of concentrations it will be seen (Table 6; Fig. 2) that the riboside is always completely metabolized, little or no residual adenosine ever being detected; this is due to the greater production of inosine. The great similarity in both cases of the amounts not converted into hypoxanthine or inosine accounts for the equal flavinogenic activity of equimolar amounts of the free base and of its riboside. The good correspondence between the fraction not metabolized and riboflavin production is also well demonstrated in Fig. 2. The reason for the greater conversion of adenosine into inosine compared with the formation of hypoxanthine from adenine is not yet obvious.

Table 5. *Efficiency of incorporation of adenine into riboflavin by* E. ashbyii

Calculated from data in Table 4 and Fig. 1 and based on total riboflavin in medium and mycelium, that in the mycelium being assumed to be 20% of that in the medium (Table 3).

Initial concn. of adenine (mM)	Incorporation of ANH into riboflavin (%)	
	Assuming incorporation into all riboflavin produced	Assuming incorporation only into riboflavin produced in excess of that found in normal cultures
0·15	118	28
0·30	105	29
0·45	76	14
0·60	55	11
1·20	70	37

Effect of acetate on adenine metabolism

The presence of acetate in the basal medium is known to inhibit flavinogenesis by *E. ashbyii* (Brown *et al.* 1955). An investigation into the effect of the presence of acetate on adenine metabolism showed that, compared with the normal medium, the situation is markedly changed (Table 7). The relative amounts of unchanged adenine and ANH are considerably increased and decreased respectively, whilst the percentage converted into hypoxanthine is greatly increased. The actual amounts are given in Fig. 3 (cf. Fig. 1) from which it can be seen that only a trace is not accounted for as either unchanged adenine or hypoxanthine. The failure of the purine to stimulate flavinogenesis in the presence of acetate is also clearly demonstrated.

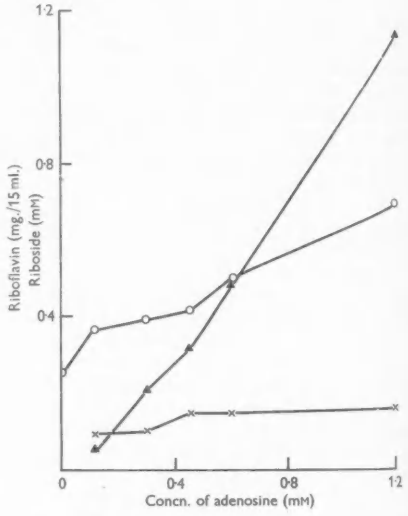


Fig. 2. Effect of concentration on the metabolism of adenosine by *E. ashbyii*. Experimental conditions were as described in Table 2. ○, Riboflavin in medium; ×, adenosine metabolized but not to inosine; ▲, inosine produced. No significant amounts of residual adenosine were noted at any concentration.

Table 6. *Influence of initial adenosine concentration on adenosine metabolism by* E. ashbyii

Experimental conditions were as given in Table 2.

Initial concn. of adenosine (mM)	Percentage converted				
	0·15	0·30	0·45	0·60	1·2
Adenosine converted into inosine	28	69	69	79	97
Adenosine metabolized but not to inosine	63	34	35	26	10
Unchanged adenosine	9	0*	0*	0*	0*

* In all these cases the sum of the first two fractions slightly exceeded 100.

Metabolism of other purines in relation to flavinogenesis in E. ashbyii

No purine metabolites were detected by paper chromatography of cultures metabolizing hypoxanthine, xanthine and guanine. Spectroscopic measurements indicated that these purines gradually disappear from the culture medium, the last to the greatest extent (Table 8). When the amounts of the purines metabolized are taken into account, their flavinogenic activities are very similar. It also appears that, at low concentrations, McNutt's view that exogenous purines are incorporated directly into riboflavin may not be correct, for the experiments recorded in Table 8 indicate 100 % incorporation into riboflavin, although no account has been taken of the riboflavin remaining in the mycelium (generally about 20 %, see Table 3).

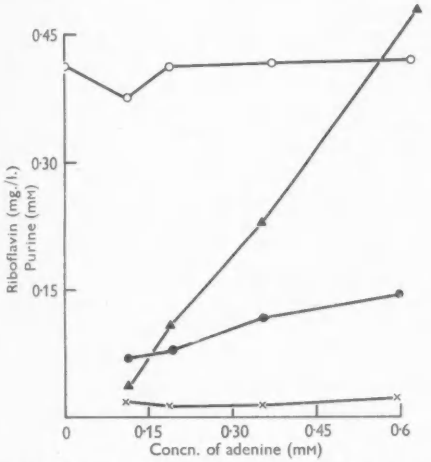


Fig. 3. Effect of acetate on the metabolism of adenine by *E. ashbyii*. Experimental details were as described in Table 7, with acetate added at a level of 0·1 M. ○, Riboflavin levels; ×, ANH; ●, hypoxanthine produced; ▲, residual adenine.

Effect of 8-azapurines on growth and flavinogenesis of E. ashbyii

The action of a number of 8-azapurines has been examined in order to see if some light would be thrown on the metabolism of purines in relation to flavinogenesis. To conserve space the results are combined and values recorded as percentages of the basal values obtaining in each particular experiment (Brown *et al.* 1955). Fig. 4 shows that (*a*) 8-aza-adenine tends to inhibit flavinogenesis specifically (e.g. at 0·125 mg./100 ml. growth is only slightly inhibited whilst riboflavin production is inhibited to the extent of 75 %) and (*b*) 8-azaguanine and 8-azahypoxanthine inhibit both growth and flavinogenesis equally, although the 8-azahypoxanthine effect is very much less marked and even at a concentration of 40 mg./100 ml. it exerts only a 40 % inhibition. The 8-aza-adenine effect is similar to that previously recorded for 8-azaxanthine (Brown *et al.* 1955), although the latter is much less toxic. A concentration of nearly 70 mg./100 ml. of 8-azaxanthine is required before slight inhibition of growth is observed. Up to its maximal solubility

Table 7. *Effect of acetate on purine metabolism in* E. ashbyii

Experimental conditions were as given in Table 2, except that 0·8 % (w/v) sodium acetate (anhydrous) was added to the basal medium.

	Percentage conversion			
Initial concn. of adenine (mM) ...	0·112	0·195	0·353	0·60
Conversion into hypoxanthine	57·6	42·1	32·2	22·1
ANH	14·6	4·9	3·8	2·6
Unchanged	27·8	53·0	64·0	75·3

Table 8. *Metabolism of xanthine, hypoxanthine and guanine by* E. ashbyii

Experimental conditions were as given in Table 2; purines were added to medium at a level of 0·37 mM.

	Purine added		
	Hypoxanthine	Xanthine	Guanine
Riboflavin content* (μg./15 ml.)	510	580	665
Purine remaining (%)	66	47	30
Purine utilized incorporated into total riboflavin (%)	107	87	96
Purine utilized incorporated into riboflavin produced above basal level (%)	21	20	25

* Basal level 315 μg./15 ml.

(0·5 mg./100 ml.) 8-aza*iso*guanine had no inhibitory effect on either growth or flavinogenesis.

With levels of 8-aza-adenine (0·125 mg./100 ml.) and 8-azaguanine (1 mg./100 ml.) which almost completely inhibit growth and flavinogenesis, the presence of the corresponding purine reverses both effects to a considerable extent (Tables 9 and 10). The inhibitory effect of 8-aza-adenine is also reversed by xanthine and, perhaps somewhat unexpectedly, by hypoxanthine. Although in the experiment recorded in Table 9 the purines were used at a level of 20 mg./100 ml., it has been found that concentrations as low as 5 mg./100 ml. are equally effective; this level of guanine is also effective against 8-azaguanine (Table 10). In none of the experiments discussed in this section was there any detectable metabolism of the 8-azapurines.

Action of 6-mercaptopurine

6-Mercaptopurine, a purine inhibitor in *Lactobacillus casei* (Elion, Hitchings & Vanderwerff, 1951), when tested up to the level of maximum solubility (2 mg./100 ml.) had no effect on growth of *E. ashbyii* but stimulated riboflavin synthesis (Table 11). The organism can presumably remove the sulphydryl group from this compound.

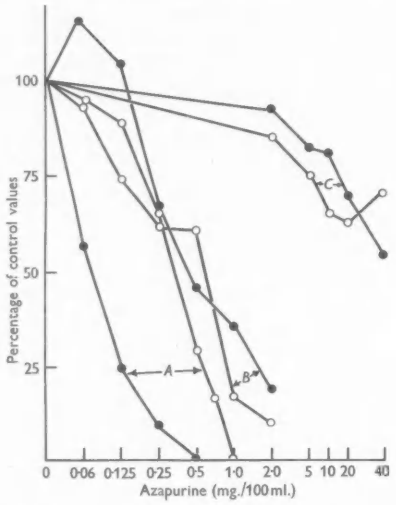


Fig. 4. Effect of certain 8-azapurines on growth and riboflavin synthesis in *E. ashbyii*. Experimental conditions were as described in Table 2; the values are recorded as the percentages of the appropriate control values. ●, Riboflavin; ○, dry weight; *A*, 8-aza-adenine; *B*, 8-azaguanine; *C*, 8-azahypoxanthine.

Table 9. *Effect of 8-aza-adenine on riboflavin production in* E. ashbyii *in the presence of excess of purines*

Experimental details were as given in Table 2; 8-aza-adenine was added at a level of 0·125 mg./100 ml., the purines at a level of 20 mg./100 ml.

Purine added to medium	Mycelium dry wt. (mg.)	Riboflavin (μg./15 ml.)
None (control)	16·2	271
8-Aza-adenine	7·3	99
Adenine	16·3	550
Xanthine	16·9	555
Hypoxanthine	15·3	475
8-Aza-adenine + adenine	16·9	434
8-Aza-adenine + xanthine	14·6	506
8-Aza-adenine + hypoxanthine	15·6	464

Table 10. *Effect of 8-azaguanine in the presence and absence of guanine on growth and flavinogenesis by* E. ashbyii

Experimental conditions were as described in Table 2. Guanine was added at a level of 5 mg./100 ml., 8-azaguanine at a level of 1 mg./100 ml.

Purine added	Mycelium dry wt. (mg.)	Riboflavin (μg./15 ml.)
None (control)	65·5	230
Guanine	60·1	435
8-Azaguanine	2·8	62
8-Azaguanine + guanine	—	305

Table 11. *Effect of 6-mercaptopurine on flavinogenesis in* E. ashbyii

Experimental details were as given in Table 2.

Concn. of 6-mercaptopurine (mg./100 ml.)	Dry wt. (mg.)	Riboflavin (μg./15 ml. of medium)
0	17·1	195
0·10	17·5	185
0·25	16·9	239
0·50	16·9	236
1·0	17·5	255
2·0	16·8	282

Action of azaserine

Azaserine, which is reported to function by preventing the incorporation of formate into purines (Skipper, Bennett & Shabel, 1954), was found to inhibit growth and flavinogenesis equally, inhibition being complete at a level of 0·1 mg./100 ml. This inhibition is not overcome by DL-serine even at a level of 80 mg./100 ml. (Table 12).

Adenine, however, at a level of 20 mg./100 ml. does stimulate growth slightly and, more significantly, exerts its usual strong flavinogenic action. This action of adenine does not allow serine to

exert its usual strongly stimulatory effect (Goodwin & Pendlington, 1954) because in the presence of azaserine a mixture of adenine and serine does not stimulate riboflavin to any greater extent than does adenine alone.

Effect of certain substituted pyrimidines

As riboflavin may be synthesized from purines via a 4:5-diaminopyrimidine after the elimination of $C_{(8)}$, it was considered that pyrimidines substituted with other groupings in the 4 and 5 positions might have an inhibitory effect on flavinogenesis. A number of such compounds, which have antimalarial activity, were examined. To conserve space, only the effects of the highest concentrations (20 μg./100 ml.; maximal solubility of most of the compounds examined) are recorded in Table 13. As the experiments were carried out at different times, the results are recorded as a percentage of the appropriate control values. It will be seen that in all cases except one, riboflavin production was inhibited to a greater extent than growth and that this was most marked in the chlorophenyl derivatives. Experiments with 2:6-diamino-4-ethyl-5-*p*-chlorophenylpyrimidine in the presence of xanthine, showed no significant inhibition of xanthine stimulation of flavinogenesis.

Table 12. *Effect of azaserine, alone and in the presence of adenine and* DL-*serine on flavinogenesis in* E. ashbyii

Experimental conditions were as described in Table 2. Azaserine was used at a level of 0·05 mg./100 ml., DL-serine at 80 mg./100 ml. and adenine at 20 mg./100 ml.

Compounds added to basal medium	Dry wt. (mg.)	Riboflavin (μg./15 ml.)
1. None (control)	11·4	403
2. Azaserine	0·9*	69
3. Serine	11·1	574
4. Adenine	10·4	552
5. Serine + adenine	11·8	740
6. Azaserine + serine	3·0	56
7. Azaserine + adenine	5·7	317
8. Azaserine + serine + adenine	5·3	308

* Too small for accurate assessment.

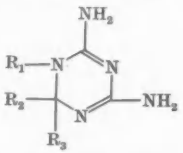
Table 13. *Effect of certain 4:5-substituted pyrimidines on growth and flavinogenesis by* E. ashbyii

Experimental details were as given in Table 2; results are expressed as percentage of appropriate control; concn. of added pyrimidine, 20 μg./100 ml.

Substituents in 2:6-diamino-4-R_2-5-R_1-pyrimidine		Dry wt. (% of control)	Riboflavin (% of control)
R_1	R_2		
3:4-Dichlorophenyl	Ethyl	84	56
4-Chlorophenyl	Ethyl	89	37
4-Chlorophenoxy	Methyl	44	43
Phenoxy	Hydrogen	87	68
4-Chlorophenyl	Methyl	110	69
4-Nitrobenzyl	Methyl	78	67

Effect of certain 1:2-*dihydro-s-triazenes*

These were examined after a report (Foley, Modest, Farber & Haley, 1955) that certain triazenes inhibited pteroylglutamic acid systems in a number of micro-organisms, and that there was a weak riboflavin inhibition (Foley & Haley, 1955) in *L. casei*. The basic structure of the compounds is



and the compounds examined had the following substituents: (*a*) R_1, phenyl; R_2, phenyl; R_3, H; (*b*) R_1, 4′-chlorophenyl, R_2 and R_3 methyl; (*c*) R_1, 3′:4′-dichlorophenyl, R_2 and R_3 methyl; (*d*) R_1, 2′-chlorophenyl, R_2 and R_3 methyl; (*e*) R_1, phenyl, $R_2 + R_3$, methyl. In no case, at the highest concentration examined, did any of these compounds show any significant growth or riboflavin inhibitory action. (We are grateful to Mr D. Treble for help with some of these experiments.)

iso*Riboflavin*

*iso*Riboflavin [5:6-dimethyl-9-(D-1′-ribityl)-*iso*alloxazine] restricts growth of riboflavin-deficient rats more than does a simple deficiency of riboflavin itself (Emerson & Tishler, 1944), but shows no signs of inhibiting the utilization of riboflavin by *L. casei* (Foster, 1944). In the present investigation no inhibition of growth or riboflavin synthesis in *E. ashbyii* by *iso*riboflavin was observed.

Antibiotics

A number of antibiotics were examined in the search for a specific inhibitor of riboflavin synthesis. None of the following had such an effect (the figures in parentheses indicate the concentration range at which the antibiotic began significantly to inhibit growth): chloramphenicol (100 mg./100 ml.), puromycin (5–10 mg./100 ml.), achromycin (50–100 mg./100 ml.) and aureomycin (0·2–0·5 mg./100 ml.). Penicillin had no effect on growth even at a level of 200 mg./100 ml.

DISCUSSION

There is now no doubt that adenine, guanine and xanthine, but not hypoxanthine or uric acid, greatly stimulate flavinogenesis in *E. ashbyii* (Tables 2 and 3) and that this stimulation is due to direct incorporation of the purines after the removal

at some stage of $C_{(8)}$ (McNutt, 1954). Two questions arise from the present investigation: (*a*) Is the large amount of riboflavin produced by *E. ashbyii* on media not supplemented with purines due to excessive production of endogenous purines? (*b*) What are the steps involved in the conversion of exogenous purines into riboflavin?

With regard to (*a*), if it is assumed that adenine and guanine are produced in excess of normal growth requirements and that this excess is funnelled into riboflavin synthesis, then most of the recorded observations can be accounted for. For example: (i) the incorporation of ^{14}C-labelled serine into riboflavin is almost completely diluted out by adenine (Goodwin & Jones, 1956); (ii) acetate, which inhibits flavinogenesis both on non-purine and purine-containing media (Brown *et al.* 1955), also strongly inhibits the metabolism of exogenous adenine (Fig. 3 and Table 7); (iii) azaserine, which probably inhibits the synthesis of purines at the stage just before the cyclization of the glycyl ribotide (Baddiley, 1955), has a general inhibitory effect both on growth and flavinogenesis with no specific action on the latter (Table 12); (iv) azaadenine and azaguanine both inhibit riboflavin synthesis to a greater extent than growth (Fig. 4). Azahypoxanthine, on the other hand, has only very slight effect on either growth or flavinogenesis, presumably because hypoxanthine is not concerned to any significant extent in the metabolism of this organism; furthermore, exogenous hypoxanthine is in fact only weakly flavinogenic. The preferential inhibition of flavinogenesis by azaxanthine (Brown *et al.* 1956) would suggest that adenine and guanine are converted into riboflavin via xanthine, which is indeed the purine most closely related to rings B and C of riboflavin.

With regard to the mechanism of conversion of exogenous purines into riboflavin, the following possibilities exist for adenine, assuming that it is deaminated at some stage before the loss of $C_{(8)}$.

1. → hypoxanthine → xanthine
2. → *iso*guanine → xanthine
3. → 2:8-dihydroxyadenine → uric acid

} → 4:5-diaminouracil

If it is assumed that the glyoxaline ring can be opened before deamination but that deamination occurs before the tricyclic ring system is formed, then the further possibilities arise:

2*a*. → *iso*guanine → 4:5:6-triamino-2-hydroxypyrimidine
3*a*. → 2:8-dihydroxyadenine ↗
4. → 4:5:6-triaminopyrimidine → 4:5-diaminouracil

It is also possible that 4:5:6-triamino-2-hydroxypyrimidine might be incorporated into the riboflavin nucleus before deamination.

The results of the present investigation allow the elimination of some of these possibilities. The first possibility can be discarded because on a molar basis hypoxanthine is very much less flavinogenic than adenine (Table 2), and in any case a considerable amount of adenine is converted into hypoxanthine (Table 4). Possible permeability effects cannot account for this difference because under comparable conditions about the same proportion of added hypoxanthine and adenine are metabolized. A small fraction of the hypoxanthine produced from adenine must obviously be converted into riboflavin, but the capacity of this route can only be very limited and most of the hypoxanthine is excreted. The second possibility could not be tested because *iso*guanine was not available. If, however, this pathway did take place one would expect aza*iso*guanine to inhibit flavinogenesis; unfortunately this is a very insoluble compound, but at the level of maximal solubility (0·5 mg./100 ml.) it had no obvious effect. It should be noted that this is a concentration above that (0·125 mg./100 ml.) at which 8-aza-adenine produces specific inhibition of flavinogenesis (Fig. 4). The preliminary conversion of adenine into 2:8-dihydroxyadenine (route 3), which occurs in animal tissues (Bendich, Brown, Phillips & Thiersch, 1950), followed by deamination to uric acid, can probably be ruled out because of the poor flavinogenic activity of uric acid (Table 2). This failure of uric acid to stimulate riboflavin synthesis cannot be due to permeability effects. That some does get into the cells is demonstrated by its slight but definite action; that being so, if it were the true precursor sufficient should have penetrated over a period of 5–10 days to produce a much greater stimulation of riboflavin synthesis. The explanation for the poor activity of uric acid is much more likely to be the same as that observed with hypoxanthine, i.e. the capacity of the route uric acid → riboflavin must be very limited. Routes 2*a*, 3*a* and 4 may be ruled out as 4:5:6-triamino-2-hydroxypyrimidine and 4:5:6-triaminopyrimidine strongly inhibit flavinogenesis (Brown *et al.* 1955). Thus adenine probably does not lose $C_{(8)}$ before its amino group but, if it is deaminated before it loses $C_{(8)}$, hypoxanthine cannot be an intermediate. Because of its structure and its strong flavinogenic activity xanthine is the most likely intermediate; it may be produced via route 2 but there is no direct evidence for this. No xanthine has ever been detected either spectroscopically or chromatographically when *E. ashbyii* is metabolizing adenine. This is, however,

no conclusive argument against its role as an intermediate. Once it is produced in the cell it may exist only for a very short time as such and be rapidly converted into riboflavin. On losing $C_{(8)}$, xanthine would be converted into 4:5-diaminouracil or a related compound before conversion into riboflavin. This is such a reactive and unstable substance that its stimulatory activity could not be assessed (Brown *et al.* 1955). It can, however, be easily converted chemically into riboflavin analogues (Birch & Moye, 1957), and recently it has been shown to be a normal metabolite of *E. ashbyii* (Goodwin & Treble, 1957). However, the problem of whether xanthine and 4:5-diaminouracil are intermediates in the conversion of adenine into riboflavin can only be finally settled with isotopically labelled adenine; this is now under investigation.

In the above discussion no consideration has been given to the question of whether purines are incorporated into riboflavin as free bases or as ribosides or ribotides. Adenosine, inosine and adenylic acid exert flavinogenic effects similar to that of adenine (Tables 2 and 3). Whatever the state of the purines when being incorporated into riboflavin, *E. ashbyii* must possess a very active system for interconverting free purines and their ribosides and ribotides.

Finally, the position of guanine is interesting because on a molar basis it is more effective than the postulated obligatory intermediate xanthine. However, calculated on the amount of purine disappearing from the medium, xanthine and guanine are equally active (Table 8), and the question which arises is why they, and hypoxanthine as well, are metabolized to different degrees. As stated previously the block in hypoxanthine metabolism is probably due to its slow rate of conversion into xanthine. A possible explanation of the guanine effect is that it is deaminated to xanthine in a region of the cell which allows faster conversion of xanthine into riboflavin than do other regions reached by exogenous xanthine.

SUMMARY

1. As stimulators of riboflavin synthesis by *Eremothecium ashbyii*, purines fall into this order of decreasing effectiveness: guanine, xanthine, adenine, hypoxanthine, uric acid. Adenosine, adenosine-3′-phosphate and inosine have the same activity as their parent compounds.

2. A spectrophotometric method has been devised to determine the amount of hypoxanthine formed from adenine in a system in which the total purine concentration is changing.

3. By this method the metabolism of adenine and adenosine by *E. ashbyii* has been studied. Adenine is converted partly into hypoxanthine, partly into riboflavin and possibly other compounds, and part remains unchanged. No xanthine could be detected. The quantitative aspects of these reactions have been studied in detail. Qualitatively adenosine behaves similarly to adenine. Quantitatively the amount converted into riboflavin is the same, but all the residue is converted into inosine; there is no unchanged adenosine remaining.

4. Acetate inhibits the metabolism of adenine.

5. Hypoxanthine, guanine and xanthine slowly disappear from the culture medium of *E. ashbyii* but no purine metabolites could be detected.

6. The effect of flavinogenesis of a number of 8-azapurines, 4:5-disubstituted pyrimidines, azaserine, 1:2-dihydro-*s*-triazenes and a number of antibiotics, has been examined.

7. A possible mechanism of conversion of purine into riboflavin has been discussed.

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A Method for the Quantitative Fractionation of Urinary 17-oxo Steroids with some Observations on Steroid Excretion During Administration of ACTH and in the Adrenogenital Syndrome

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Many methods have been described for the estimation of the separate fractions of urinary 17-oxo steroids. However, the earlier methods used hot-acid hydrolysis to cleave the 17-oxo steroid conjugates and it is well known that this procedure causes considerable destruction of steroids and the formation of artifacts (Dorfman & Ungar, 1953). Most of the earlier methods also made no attempt to resolve the individual 17-oxo steroids possessing an oxygen function attached to $C_{(11)}$ of the steroid nucleus. More recently two methods have been described which do this (Rubin, Dorfman & Pincus, 1954; Kellie & Wade, 1956*a*). In the first of these the urine is hydrolysed with hot acid and the 3α-hydroxy ketonic fraction separated into its constituents by repeated paper chromatography in different systems. Artifacts formed during the hydrolysis and Girard separation are individually estimated and the quantities obtained added to the assays of their presumed precursors. Kellie & Wade hydrolyse an extract of urinary conjugates by mild methods and then separate the liberated 17-oxo steroids by gradient elution on a column of alumina.

The method to be described here was devised particularly to fractionate the individual 17-oxo steroids of urines containing low concentrations of these compounds.

NOMENCLATURE

The trivial names androsterone, aetiocholanolone, dehydro*epi*androsterone, 11-oxoandrosterone, 11-oxoaetiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxyaetiocholanolone are used for 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one, 3β-hydroxyandrost-5-en-17-one, 3α-hydroxy-5α-androstane-11:17-dione, 3α-hydroxy-5β-androstane-11:17-dione, 3α:11β-dihydroxy-5α-androstan-17-one and 3α:11β-dihydroxy-5β-androstan-17-one respectively throughout this paper.

EXPERIMENTAL

Apparatus

The glass columns for alumina chromatography were 17 cm. long and had an internal diameter of 4 mm. They were tapered at the lower end and a bulb of 100 ml. capacity was fused to the upper end. For paper chromatography, Shandon all-glass tanks were used.

Methods

The flow sheet (Fig. 1) shows the overall plan of the procedure.

Hydrolysis and extraction. A volume of urine is taken which routine 17-oxo steroid analysis (Norymberski, Stubbs & West, 1953) has shown to contain 2 mg. of crude 17-oxo steroid. This specimen is extracted by the method of Edwards, Kellie & Wade (1953) and the ether–ethanol solution evaporated to give a residue containing the urinary 17-oxo steroid conjugates. This residue is dissolved in water (35 ml.), 3 ml. of M-acetate buffer, pH 4·3, added and the solution incubated with β-glucuronidase (20 000 units) at 37°. The batch of β-glucuronidase used in this laboratory was prepared from the visceral humps of limpets (*Patella vulgata*) by a slight modification of an early method (Mills, 1948) and had an activity of 20 000 units/ml. After incubation for 48 hr. the solution is extracted with ether (2 × 30 ml.) and the combined ether extracts are washed with water (1 × 10 ml.), 2N-NaOH (1 × 10 ml.) and water (2 × 10 ml.). The washed ether extract is evaporated to give a residue containing the 17-oxo steroids liberated from the glucuronides. The aqueous solution, together with the first water washing, is placed in the upper vessel of a liquid–liquid extractor and 10N-H_2SO_4 (2 ml.) added. Ether (100 ml.) is placed in the lower vessel of the extractor, which also contains a few chips of marble to neutralize any acid carried down, and the extraction is maintained by refluxing the ether for 3 days.

The ether extract is then washed with water (1 × 15 ml.), 2N-NaOH (1 × 15 ml.) and water (2 × 15 ml.), and evaporated to dryness to give a residue containing the 17-oxo steroids liberated from conjugation with H_2SO_4. The first water washing is added to the aqueous solution, which is then treated with 14 ml. of 11N-HCl and heated on a boiling-water bath for 30 min. After cooling, the solution is extracted with ether (2 × 30 ml.) and the combined ether extracts are washed with 2N-NaOH (2 × 10 ml.) and water (2 × 10 ml.) and then evaporated to dryness.

Portions of the dried residues from each hydrolytic procedure containing 5–50 μg. of 17-oxo steroid are taken for Zimmermann assay. The method employed for assaying here, and subsequently, is essentially that of Edwards *et al.* (1953). The dry fraction is treated in turn with 1% *m*-dinitrobenzene in ethanol (0·1 ml.) and ethanolic 2·5N-KOH (0·05 ml.). After 1 hr. at 25° the mixture is diluted with 2·5 ml. of ethanol and the optical density of the mixture is measured with Ilford spectrum green (no. 604) and vic.[let] (no. 601) filters in a Spekker absorptiometer with cells having an optical depth of 1 cm. and a volume of 2·5 ml.

Fig.

Girard separation and adsorption chromatography. The extracts obtained by the three methods of hydrolysis were combined, dried and separated into ketonic and non-ketonic fractions by the Pincus & Pearlman (1941) modification of the Girard procedure. Since some acetylation of the free hydroxide groups takes place under these conditions, it is necessary to hydrolyse the acetates which have been formed so that subsequent fractionation is simplified. The evaporated ketonic fraction is therefore dissolved in methanol (1 ml.), 0·2 ml. of an aqueous solution of $KHCO_3$ containing 50 mg./ml. is added and the mixture allowed to stand overnight at 37°. The solution is then partitioned between ether (30 ml.) and water (7 ml.) and the aqueous phase extracted with a further 10 ml. of ether. The combined ether extracts are washed with water (2 × 5 ml.) and evaporated to dryness.

The dry ketonic fraction is separated into less polar and more polar fractions by a simple chromatogram on alumina. The column is prepared in the following way. A small plug of cotton wool is inserted into the lower (tapered) end of the glass column. A slurry of alumina (1·5 g., Savory and Moore, Brockmann activity II–III) in 4–5 ml. of benzene is poured into the top of the bulb and the column gently tapped while the alumina settles down. The ketonic fraction is dissolved in benzene (5 ml.) and 4 ml. of this solution carefully run on to the top of the column; a further 0·1 ml. portion of the benzene solution is taken for Zimmermann assay. The steroids are eluted successively with 50 ml. of benzene containing 0·3% ethanol (fraction 1) and 20 ml. of benzene containing 10% ethanol (fraction 2). Development of the column may be considerably hastened by the application of pressure (100 mm. Hg). A 2·5 l. bottle (e.g. a Winchester) fitted with a manometer and a hand bellows forms a convenient air reservoir and can provide a stable source of pressure for up to six columns.

Fraction 1 contains those 17-oxo steroids which do not possess an oxygen function in the $C_{(11)}$ position and fraction 2 contains those which do. Portions of the two fractions may be taken for Zimmermann assay: one-thirtieth of the less polar fraction and one-tenth of the more polar fraction.

Paper chromatography. Whatman no. 2 filter paper is cut as shown in Fig. 2. The less polar fraction (1) is run in a Bush (1952) *A* system (light petroleum–methanol–water, 5:4:1, by vol.) at room temperature with standard

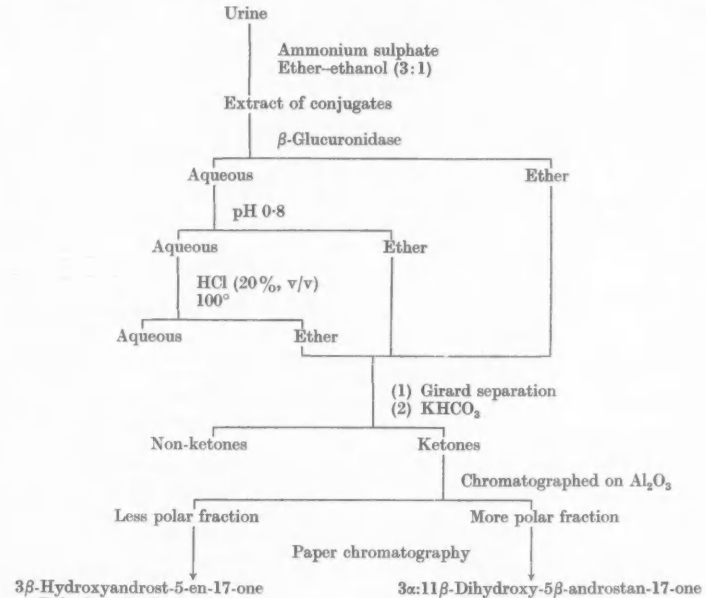

Fig. 1. Scheme for fractionating urinary 17-oxo steroids. The individual steroids isolated in the less polar and more polar fractions are listed in the order in which they appear on their respective paper chromatograms, compounds with the greatest mobility being at the bottom of the list.

reference compounds on lanes 1, 3 and 5. Lanes 1 and 5 have only aetiocholanolone, but lane 3 has, in addition, dehydro*epi*androsterone, androsterone and 5α-androstane-3:17-dione. A mixture containing 20 μg. of each of these four standards in 20 μl. of ethyl acetate is used.

The more polar fraction (2) is run in a system consisting of light petroleum–toluene–methanol–water (667:333:800:200, by vol.) at 37°. Reference standards 11β-hydroxyaetiocholanolone, 11β-hydroxyandrosterone and 11-oxoaetiocholanolone are run in lane 3 and 11β-hydroxyandrosterone only in lanes 1 and 5. It is desirable to apply 150–200 μg. of the unknown mixture of 17-oxo steroids to the 3 cm. wide lanes. In practice this usually means taking one-quarter of the less polar fraction or all the more polar fraction. Standards and unknowns are applied to the strips with 200 μl. graduated pipettes and simultaneously dried by a jet of nitrogen directed at the under side of the strip. The papers are then hung in the tanks overnight to equilibrate and run the following day for 6 hr. When the papers have been removed from the tanks and dried, the 3 mm. wide strips marked on the middle of lanes 2 and 4 are cut out and, together with lanes 1, 3 and 5, dipped in a mixture of equal volumes of 2% *m*-dinitrobenzene in ethanol and ethanolic 2·5 N-KOH. The strips are allowed to drain for 30 sec. before being laid on a horizontal sheet of glass. After 5–10 min. at room temperature they are transferred to an oven at 90° for 3 min.: this procedure gives minimal background colour.

Regions of the remainder of the 3 cm. lanes corresponding to the spots obtained on the test portion are then cut out and placed in stoppered test tubes overnight with 2·5 ml. of ethanol. Two 1 ml. portions from each of these solutions are evaporated to dryness and assayed in the usual way. If there is reason to doubt the identity of any Zimmermann-reacting spot on the test strip, the two coloured solutions obtained after assaying the corresponding eluted zone are combined and acidified and the 17-oxo steroid–*m*-dinitrobenzene complex extracted as described by Kellie & Smith (1956). The extract may then be rechromatographed on paper together with the appropriate 17-oxo steroid–*m*-dinitrobenzene standard in the same system as was used for the corresponding free 17-oxo steroid.

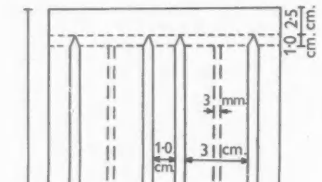

Fig. 2. Plan of paper for quantitative chromatography of 17-oxo steroids. The full lines indicate the shape in which the paper is cut before chromatography and the interrupted lines are for reassessment. The 3 mm. strips (indicated by interrupted lines) in the middle of the 3 cm. lanes are cut out after chromatography and, together with the standard strips 1, 3 and 5, treated with Zimmermann reagents.

11-Oxoaetiocholanolone and its 5α-epimer do not separate well in the system used for resolving the more polar fraction. If the amount of 5β-epimer is large compared with the 5α-epimer the two zones may be confluent, making it impossible to distinguish the separate components. In such cases the complete section of the 3 cm. lane corresponding to the two 3α-hydroxyandrostane-11:17-diones is cut out and eluted in the usual way. One 2 ml. portion is removed from the solution, evaporated and the residue so obtained applied to the start line of a 3 cm. lane. The two standard substances are applied to neighbouring 1 cm. wide lanes and the chromatogram is developed in a Bush *A* system at room temperature for 24 hr.; this procedure gives very good separation.

RESULTS

Recovery of crystalline steroids in the Girard separation

In the fractionation of the urinary 17-oxo steroids from the cases reported in this paper (Tables 2 and 3), the hydrolysis of any acetates formed during the Girard separation was effected by dissolving the ketonic fraction in ethanolic 0·5 N-KOH and allowing the mixture to stand at room temperature overnight. However, in two experiments in which 11-oxoaetiocholanolone (98 μg.) was added to the specimens of urine from two patients with hypopituitarism, recoveries of less than 40% were obtained when the complete fractionation procedure was followed. The loss was traced to the hydrolysis with ethanolic KOH after the Girard separation. Table 1 shows the results of an experiment in which 200 μg. quantities of six crystalline 17-oxo steroids were individually put through the Girard separation, after which the ketonic fractions were divided into three equal parts. Part (*A*) was chromatographed on paper and in three cases this gave rise to two Zimmermann-reacting spots, the more mobile of which was probably 17-oxo steroid acetate. Part (*B*) was hydrolysed overnight at room temperature with ethanolic 0·5 N-KOH before being chromatographed; part (*C*) was hydrolysed overnight with $KHCO_3$ in aq. methanol at 37° and then chromatographed on paper. In every case parts (*B*) and (*C*) gave only one Zimmermann-reacting spot, although part (*B*) of the 11-oxoaetiocholanolone and 11-oxoandrosterone series gave a non-specific grey-blue chromogen 8 cm. in front of the 17-oxo steroid zone.

Table 1. *Recovery of crystalline 17-oxo steroids*

Recoveries recorded after Girard separation followed by (*A*) paper chromatography, (*B*) hydrolysis with ethanolic 0·5*N*-KOH and paper chromatography, (*C*) hydrolysis with $KHCO_3$ in aq. methanol and paper chromatography.

	Recovery (%)			
	(*A*) Girard separation		(*B*) Girard separation + KOH hydrolysis	(*C*) Girard separation + $KHCO_3$ hydrolysis
Compound	Main spot	Mobile spot		
Androsterone	64	7	70	85
Aetiocholanolone	61	—	79	86
Dehydro*epi*androsterone	71	5	77	82
11-Oxoandrosterone	68	—	13	79
11-Oxoaetiocholanolone	81	5	11	87
11β-Hydroxyandrosterone	87	—	96	90

The recovery of these two compounds was very low after hydrolysis with KOH, but not when $KHCO_3$ was used. There was no significant difference in the recoveries of the other compounds by the three procedures described above and it therefore seems justifiable to use hydrolysis with $KHCO_3$ as the standard method.

Recovery from alumina chromatograms

In chromatography on alumina the most polar of the major urinary 17-oxo steroids without an oxygen attached to $C_{(11)}$ is aetiocholanolone. Recovery experiments were therefore carried out with an extract of hydrolysed urine divided into three equal parts, to one of which was added aetiocholanolone and to another 11-oxoaetiocholanolone. The mean recovery for the two compounds in the appropriate fractions was essentially quantitative, being 97 % for aetiocholanolone and 103 % for the 11-oxo analogue. Any considerable departure of the alumina from this performance would, of course, be revealed on the subsequent paper chromatography, i.e. by Zimmermann-reacting material moving more slowly than dehydro*epi*androsterone in the paper chromatography of the less polar fraction or by a spot near the solvent front in the more polar system.

Recovery from paper chromatograms

The recovery of crystalline androsterone (80 μg.) and dehydro*epi*androsterone (52 μg.) chromatographed together on 3 cm. wide lanes in the Bush *A* system and eluted in the way described in the method was tested in a series of six experiments. After development of the chromatogram the zones were eluted on the basis of the distances run by the standards androsterone, aetiocholanolone and dehydro*epi*androsterone and assayed in duplicate. For androsterone the mean recovery was $87 \pm 2{\cdot}8$ % (S.D.) and for dehydro*epi*androsterone $92 \pm 3{\cdot}6$ % (S.D.). Aetiocholanolone, which travels between these two compounds in this system, was not applied to the recovery lanes, so that any material found in this zone must have been derived from the neighbouring androsterone and dehydro*epi*androsterone zone and is therefore a measure of the inefficiency of separation. The percentage of the total 17-oxo steroids applied which was found in this region was $3{\cdot}7 \pm 1{\cdot}2$ % (S.D.).

Recovery of added steroid through the complete method

In addition to the two 11-oxoaetiocholanolone recovery experiments already reported, three other overall recovery experiments were done. In the first 11β-hydroxyaetiocholanolone (91 μg.) and sodium dehydro*epi*androsterone sulphate (110 μg.) were added to one-sixth of a 24 hr. urine collection from a patient with Addison's disease. This specimen was put through the complete fractionation procedure in parallel with a similar portion to which no steroid had been added. The recoveries of these two compounds in their appropriate fractions were 73 and 72 % respectively. In the second and third recovery experiments, androsterone (250 μg.) was added to specimens of urine from two patients with hypopituitarism. The recoveries of the added steroid in the androsterone regions of the paper chromatograms were 75 and 82 % when compared with their respective control specimens.

Normal values

Table 2 shows the results of fractionating the urinary 17-oxo steroids in two normal men and six normal women. The proportion of the crude total 17-oxo steroids hydrolysed by β-glucuronidase varied from 58 to 86 %, and continuous extraction at pH 0·8 released 12–34 % and hot acid 2–13 % of the total. These figures agree well with those reported by Kellie & Wade (1956*b*). However, the colours obtained when performing the Zimmermann reaction with the fractions liberated by the final hot-acid hydrolysis were very impure, usually showing a greater absorption with the violet filter than with green. The apparent 17-oxo steroid content of the ketonic fractions represent from 57 to 71 % of the crude total fractions. This range is narrower than

Table 2. *Fractionation of the urinary* 17*-oxo steroids in normal human individuals*

All amounts are in mg./24 hr. and for individual compounds they are corrected for the colour equivalent of the compound concerned in the Zimmermann reaction. F, female; M, male.

Case no.	1	2	3	4	5	6	7	8
Sex	F.	F.	F.	F.	F.	F.	M.	M.
Age (years)	28	21	23	30	26	22	25	40
Hydrolysed by β-glucuronidase	10·5	7·4	10·4	9·0	8·0	10·2	14·7	10·5
Hydrolysed at pH 0·8	3·9	1·0	1·7	3·2	2·4	2·4	8·8	5·2
Hydrolysed by HCl at 100°	0·6	0·2	0·6	0·9	1·5	0·9	2·1	1·0
Total	15·0	8·6	12·7	13·1	11·9	13·5	25·6	16·7
Ketonic fraction	8·6	5·3	8·2	7·6	7·5	8·6	15·0	11·9
Less polar fraction	7·8	5·1	8·4	6·5	6·5	6·4	14·0	10·2
More polar fraction	1·6	1·3	1·3	1·2	1·4	1·6	1·6	1·3
Unidentified mobile fraction	—	—	—	0·1	0·2	0·2	0·3	—
Dione fraction	—	—	0·1	—	—	—	—	—
Androsterone	1·9	2·2	3·4	2·8	2·9	2·3	4·6	4·0
Aetiocholanolone	2·3	1·4	2·5	2·0	1·5	3·0	3·0	2·7
Dehydro*epi*androsterone	1·1	0·1	0·2	0·3	0·4	0·3	2·7	2·4
11-Oxoaetiocholanolone	0·13	0·10	0·04	0·16	0·17	0·16	0·22	0·12
11β-Hydroxyandrosterone	0·85	0·75	0·82	0·61	0·85	0·54	1·40	0·46
11β-Hydroxyaetiocholanolone	0·47	0·21	0·21	0·23	0·36	0·51	0·47	0·41

that reported by Dobriner, Kappas, Rhoads & Gallagher, 1953 and by Kappas & Gallagher, 1955.

Of the individual 17-oxo steroids the relatively low excretion of dehydro*epi*androsterone in five of the six women should be noted. The ratio of androsterone to its 5β isomer covers the range 2:1 to 0·8:1. The two isomeric androstane-3:17-diones (5α- and 5β-) are not separated in this method but only in case 3 was any material found in this zone. However, four other individuals (cases 4–7) had very small amounts of unknown material which had greater mobility than the diones in this system.

The 17-oxo steroid found in greatest quantity in the more polar group is, in every case, 11β-hydroxyandrosterone, the daily amounts excreted varying from 0·46 to 1·4 mg. The amounts of 11-oxoaetiocholanolone found in this normal series are low compared with the quantities reported by some workers (Rubin *et al.* 1954; Kappas & Gallagher, 1955; Kellie & Wade, 1956*b*). This is presumably due to some destruction of this compound taking place during hydrolysis with ethanolic KOH which was used at this time in place of hydrolysis with $KHCO_3$ in aq. methanol, now the standard procedure. In the reports by other workers referred to above this loss did not arise either because the Girard separation was not used (Kellie & Wade, 1956*b*) or because no attempt was made to hydrolyse acetates formed during the Girard reaction (Rubin *et al.* 1954; Kappas & Gallagher, 1955). It would appear from the two experiments referred to earlier, in which 11-oxoaetiocholanolone was recovered from urine by the fractionation method incorporating hydrolysis with ethanolic KOH, that the actual values for the excretion of this 17-oxo steroid should be several times those reported here.

Effect of stimulation with adrenocorticotrophic hormone

Table 3, columns 1 and 2, shows the pattern obtained by fractionating the 17-oxo steroids in a 14-year-old boy before and during administration of adrenocorticotrophic hormone (ACTH) gel injected intramuscularly. Treatment causes the greatest changes in the excretion of dehydro*epi*androsterone and 11β-hydroxyaetiocholanolone, both of which show 15-fold increases.

Separate fractionation of the 17*-oxo steroids liberated by the different hydrolytic procedures*

The remaining columns of Table 3 show the results obtained on fractionating the 17-oxo steroids released in two patients after applying the successive methods of hydrolysis. Patient 10 was a girl (13 years old) with the adrenogenital syndrome due to congenital adrenal hyperplasia; she was at this time receiving no hormone treatment. Patient 11 had Klinefelter's syndrome.

The extract containing the urinary-steroid conjugates from case 10 was hydrolysed in the usual way, first with β-glucuronidase, then at pH 0·8 during continuous extraction with ether and finally by boiling with hot mineral acid. However, the three extracts obtained from these procedures were not combined before the Girard separation but were individually put through the entire fractionation procedure, the results being shown in columns 3, 4 and 5. Similarly, column 6 shows the composition of the fraction liberated by the action of β-glucuronidase on the extract from case 11, and column 7 shows the additional amounts released by the continuous extraction with ether at pH 0·8. Column 8 shows the residual amounts obtained by hot acid.

Table 3. *Urinary 17-oxo steroid excretion during treatment with adrenocorticotrophic hormone (ACTH) and in the adrenogenital syndrome*

Columns 3–8 show the amounts released by the successive application of the three hydrolytic procedures in cases 10 and 11. All amounts are in mg./24 hr. and for individual compounds they are corrected for the colour equivalent of the compound concerned in the Zimmermann reaction.

Column no.	1	2	3	4	5	6	7	8
Case no.	9	9	10	10	10	11	11	11
Treatment	Nil	ACTH	Nil	Nil	Nil	ACTH	ACTH	ACTH
Hydrolysed by β-glucuronidase	4·7	31·0	80·8	—	—	60·6	—	—
Hydrolysed at pH 0·8	1·8	24·0	—	15·7	—	—	11·7	—
Hydrolysed by HCl at 100°	0·7	8·0	—	—	7·0	—	—	4·0
Total	7·2	63·0	—	—	—	—	—	—
Ketonic fraction	5·0	40·4	—	—	—	41·7	2·9	—
Less polar fraction	3·2	26·2	37·8	7·4	0·6	27·3	1·5	0·48
More polar fraction	1·0	15·4	20·7	1·7	0·4	11·3	1·3	0·05
Dione fraction	0·02	0·3	—	0·7	—	—	—	—
Androsterone	1·3	6·2	21·1	1·0	0·06	10·3	0·2	—
Aetiocholanolone	0·9	5·7	12·1	0·7	0·05	16·5	0·4	—
Dehydro*epi*androsterone	0·4	6·8	0·3	2·8	0·06	0·4	0·6	—
11-Oxoaetiocholanolone	0·13	0·8	2·2	0·1	0·1	0·5	0·2	—
11β-Hydroxyandrosterone	0·71	7·1	22·4	1·0	0·1	2·6	0·6	—
11β-Hydroxyaetiocholanolone	0·43	7·1	4·4	0·2	0·1	7·7	0·7	—

The most notable feature of these results from the standpoint of methodology is that whereas in patient 10 the crude 17-oxo steroids obtained by hot-acid hydrolysis (column 5) form 7 % of the total obtained by the successive application of all three hydrolytic procedures (column 3 + 4 + 5), after the removal of the non-ketones by Girard separation this proportion is only 1·5 %. In patient 11 the corresponding proportions are 6 and 1·3 %. β-Glucuronidase released 78 % in patient 10 and 80 % in patient 11 of the total crude 17-oxo steroids. However, the results show that the extracts obtained by β-glucuronidase hydrolysis are relatively pure so that the predominance of the glucuronide mode of conjugation is greater than these percentages indicate.

In these two patients only one compound is found in greater amount in the fraction hydrolysed at pH 0·8 than in the β-glucuronidase fraction, namely dehydro*epi*androsterone. The small amount of this compound found in the fraction hydrolysed by β-glucuronidase (cf. Kellie & Wade, 1956*b*) may have been excreted as the glucuronide or they may have been present in the urine in the free state. Control experiments with sodium dehydro*epi*androsterone sulphate, prepared synthetically, showed that the enzyme preparation used did not liberate the free steroid under the standard conditions.

In case 10 the large amounts of more polar 17-oxo steroids excreted should be noted; especially significant is the excretion of 11β-hydroxyandrosterone which, together with its 11-deoxy analogue, forms two-thirds of the total. In spite of the level of 23·4 mg. of 11β-hydroxyandrosterone/24 hr. released by β-glucuronidase and hydrolysis at pH 0·8 only a further 0·1 mg. is liberated by hot acid. This contrasts with the evidence of Lieberman, Mond & Smyles (1954) for the existence of a conjugate of this compound which is not hydrolysed by enzyme in 60–65 hr. nor by mild acid treatment.

DISCUSSION

The method described is lengthy in that almost 2 weeks is required to complete the fractionation of one specimen. However, this is largely because of the mild methods of hydrolysis used: the manipulation time required is not inordinately great. One person can begin the fractionation of four specimens each week, so that during the hydrolysis of any one batch of four the subsequent purification of the preceding batch is being carried forward simultaneously. It is apparent from the results of the two experiments (cases 10 and 11), in which the residues obtained by the final hot-acid hydrolysis were further fractionated on their own, that this hydrolytic stage is unnecessary and may be omitted.

It was recommended earlier that a volume of urine containing 2 mg. of crude 17-oxo steroids should be taken for analysis. In fact the sensitivity of the method is greater than might be inferred from this figure, which was selected for normal cases in which the amount in the more polar fraction is less than one-quarter of that in the less polar fraction. In many cases in which the excretion of 17-oxo steroids is low (e.g. in children, old people, patients with hypopituitarism), the two fractions are more nearly equal and a sample containing as little as 0·5 mg. may be sufficient for analysis. The fairly extensive purification involved in this method

makes it possible to fractionate the 17-oxo steroids in such specimens without difficulty, since the final extracts for paper chromatography are relatively free from pigments and non-ketonic chromogens. In view of the comparatively large number of steps in the method with the inevitable losses associated with each stage in the purification, the overall recoveries of added crystalline 17-oxo steroids would appear to be reasonable and justified by the increased specificity which is ensured.

Ethanolic potassium hydroxide was the method initially chosen to hydrolyse any 17-oxo steroid acetates formed in the Girard separation, for two reasons: first, because this method has been used by other workers for splitting 17-oxo steroid acetates (Edwards *et al.* 1953), and secondly, because preliminary experiments with dehydro*epi*androsterone acetate indicated complete hydrolysis at room temperature overnight without loss of steroid or formation of artifacts. However, poor recoveries of 11-oxoaetiocholanolone from urine led to the performance of the comparative experiment, the results of which were shown in Table 1, and resulted in the adoption of potassium bicarbonate in aqueous methanol for hydrolysis; in this way therefore it should be possible to get a quantitative estimate of all the major urinary 17-oxo steroids.

The presence of large amounts of 17-oxo steroids in the more polar group of case 10 is of interest because of the light it sheds on the nature of the biochemical abnormality in the adrenogenital syndrome. The 17-oxogenic steroids (Gibson & Norymberski, 1954) excreted by this patient were 43 mg./day, a figure which could be accounted for by the level of pregnanetriol (5β-pregnane-3α:17α:20α-triol), which by the method of Bongiovanni & Clayton (1954) was 45 mg./day. Urinary 17-hydroxycorticosteroids determined by the method of Reddy, Jenkins & Thorn (1952) were normal (5·1 mg./day). This picture of the excretion of $C_{(21)}$ metabolites of the adrenal steroids by this patient was confirmed by the chromatography of the 17-oxogenic steroids on alumina. Only 26 % of the 17-oxogenic steroids were found to have an oxygen atom attached to $C_{(11)}$, whereas in the normal individual 11-oxygenated 17-oxogenic steroids predominate. There is therefore a contrast between the high proportion of 11-oxygenation among the C_{19} steroids and the low proportion among the C_{21} steroids. Jailer (1953) has suggested that in virilizing adrenal hyperplasia there is a metabolic block in the conversion of 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3:20-dione) into cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione) and that this intermediate is metabolized by alternative pathways, e.g. to pregnanetriol and 17-oxo steroids. Dorfman (1954) has collected evidence which shows that whereas 11-oxygenated C_{21} compounds with the Δ^4-3 keto grouping are metabolized to C_{19} compounds, chiefly of the 5β-series, adrenosterone (androst-4-ene-3:11:17-trione) yields mainly 5α-urinary metabolites. The patterns of C_{21} and C_{19} steroid excretion in this patient conform to this concept well. Thus the very large amounts of 11-hydroxylated 17-oxo steroids are mainly the 5α-compound and would appear to be derived from a C_{19} precursor with a Δ^4-3 keto grouping. The high ratio of androsterone to its 5β-analogue found here has been reported also by Wilkins, Bongiovanni, Clayton, Grumbach & van Wyk (1955), in one of their cases with this syndrome and also by Pond (1954). This high 5α:5β ratio indicates that if any considerable proportion of the urinary 17-oxo steroids are derived from 17-hydroxyprogesterone then fission of the side chain at $C_{(17)}$ occurs before reduction of the A ring of the steroid nucleus.

SUMMARY

1. A method is described for the analysis of the major urinary 17-oxo steroids.

2. An extract containing the urinary 17-oxo steroid conjugates is hydrolysed first with β-glucuronidase, then by continuous extraction with ether at pH 0·8. The liberated 17-oxo steroids are purified by application of the Girard treatment and the ketonic fraction is treated with potassium bicarbonate in aqueous methanol to hydrolyse any acetates formed in the Girard separation. The product is then separated into two fractions by chromatography on a column of alumina. The first fraction eluted contains those 17-oxo steroids without an oxygen attached to $C_{(11)}$ and the second fraction contains the 17-oxo steroids with a $C_{(11)}$ oxygen function. These two fractions are resolved into their component 17-oxo steroids by paper chromatography, the final estimation being made by applying the Zimmermann reaction to residues obtained after eluting the individual steroids from the paper.

3. Recovery of added 17-oxo steroid through the complete procedure is 70–80 %.

4. Evidence is presented to show the effectiveness of the hydrolytic procedures.

5. Normal values for six females and two males are given, and also values after stimulation with adrenocorticotrophic hormone, and for one case of the adrenogenital syndrome.

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The Synthesis of *N*-Acetylneuraminic Acid

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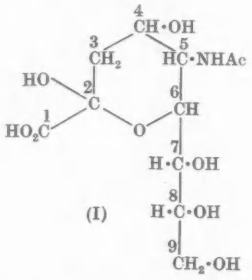
The term neuraminic acid is now reserved for a hypothetical amino sugar whose derivatives are found combined in many animal tissues, secretions and excretions. These derivatives of natural occurrence, which are known as sialic acids (for nomenclature see Blix, Gottschalk & Klenk, 1957), have acyl groups bound to the amino group and sometimes also to a hydroxyl group of the parent neuraminic acid; the commonest substituent is acetyl. The first sialic acid to be crystallized was isolated from bovine-submaxillary-gland mucin by Blix (1936); it is now known to be an *ON*-diacetylneuraminic acid (Blix, Lindberg, Odin & Werner, 1956). Crystalline *N*-acetylneuraminic acid was first prepared from the same mucin by Klenk & Faillard (1954), the *O*-acetyl group having been lost when the mucin's acidity was used to catalyse its own hydrolysis. Later, the influenza-virus enzyme neuraminidase (Gottschalk, 1951, 1957*a*) was used to release *N*-acetylneuraminic acid from human urinary mucoprotein (Klenk, Faillard & Lempfrid, 1955). Since then, the acid has been isolated from pseudomyxomatous cysts (Odin, 1955*a*), human-serum proteins (Odin, 1955*b*; Svennerholm, 1956; Böhm, Ross & Baumeister, 1957), human-cervix-uteri mucus (Odin, 1955*c*), human meconium (Odin, 1955*b*), human gangliosides (mucolipids) (Blix & Odin, 1955; Svennerholm, 1956), human lipid-free brain tissue (Svennerholm, 1956), human liver (Martinsson, Raal & Svennerholm, 1957), stroma of human erythrocytes (Klenk & Lempfrid, 1957), human milk (Svennerholm, 1956), fetuin (Klenk & Faillard, 1957), boar-seminal mucin (Odin, 1955*c*), ovine-submaxillary-gland mucin (Blix *et al.* 1956), and from ovomucin (Odin, 1955*c*).

In mucoproteins and mucolipids, the sialic acid forms part of a very large molecule, but Kuhn & Brossmer (1956*a*) have isolated from cow colostrum a trisaccharide in which *ON*-diacetylneuraminic acid is bound glycosidically to lactose. Gynaminic acid from the non-dialysable fraction of human milk is identical with *N*-acetylneuraminic acid (Zilliken, Braun & György, 1955). Sialic acid of porcine-submaxillary-gland mucin and of the stroma of erythrocytes from horse, ox and swine was shown to be *N*-glycolloylneuraminic acid (Blix *et al.* 1956; Klenk & Uhlenbruck, 1957). Horse-serum lactaminic acid as prepared by Yamakawa & Suzuki (1955) is almost certainly the methyl ester of *N*-acetylneuraminic acid.

When Klenk's (1941) original method of heating at 100° with 5% methanolic hydrogen chloride is used to split sialic acid from mucoids, the methylglycoside of neuraminic acid is obtained, all acyl groups being lost by alcoholysis. This glycoside has been prepared from gangliosides (Klenk, 1941),

amyloid (Klenk & Faillard, 1955), stroma protein of bovine erythrocytes (Klenk & Stoffel, 1956), glycolipid of horse erythrocytes (Yamakawa & Suzuki, 1951, 1952), horse-submaxillary-gland mucin (Klenk & Uhlenbruck, 1956), and from all sialic acids (Blix *et al.* 1956). In other examples where a crystalline product was not isolated, Bial's orcinol reagent and the direct Ehrlich reaction have been used as specific agents for detecting sialic acid (Werner & Odin, 1952; Gottschalk, 1955*a*; Kuhn & Brossmer, 1956*a*; Böhm & Baumeister, 1956; Svennerholm, 1957). Sialic acid is notably absent from mucopolysaccharides containing hexuronic acid (Gottschalk, 1955*a*).

The first degradation product of known structure from a sialic acid was obtained when pyrrole-2-carboxylic acid was isolated from the alkaline hydrolysate of homogeneous mucoprotein of bovine submaxillary gland and from human urinary mucoprotein (Gottschalk, 1953, 1955*a*). The product released from these mucoproteins by the action of neuraminidase from influenza virus or from *Vibrio cholerae* gave the same acid upon very mild alkali treatment (Gottschalk, 1954), and so also did *N*-acetylneuraminic acid (Klenk & Faillard, 1954). These findings, together with a reinterpretation of the data then available from the Laboratories of Blix and Klenk (for review see Gottschalk, 1956) on the properties and composition of sialic acids, and the observation that pyrrole-2-carboxylic acid is obtained by alkaline condensation of D-glucosamine with pyruvic acid, led us to propose the structure (I) for *N*-acetylneuraminic acid (at the time without indication of the stereochemistry) (Gottschalk, 1955*b*).



(I)

It was very desirable to prove this structure by synthesis; and since the molecule expressed by formula (I) may be regarded as a product of aldol condensation between an *N*-acetylhexosamine and pyruvic acid, a plan for the work was easily formed. We chose *N*-acetyl-D-glucosamine as the hexosamine component, chiefly because of its availability. When the synthesis was almost completed, the degradation of *N*-acetylneuraminic acid to *N*-acetyl-D-glucosamine, carbon dioxide and an unknown C_2 fragment by heating with pyridine and nickelous acetate (Kuhn & Brossmer, 1956*b*), and the fission of *N*-acetylneuraminic acid into *N*-acetyl-D-glucosamine and pyruvic acid by alkali (Zilliken & Glick, 1956) and apparently by an enzyme of *Vibrio cholerae* (Heimer & Meyer, 1956), helped to confirm the structure which we were trying to synthesize and showed that the first stereochemical step had been taken in the right direction.

The initial experiments on condensation of pyruvic acid with *N*-acetyl-D-glucosamine gave, however, no product responding to Bial's orcinol test; the condensation of glucosamine with pyruvic acid, which gives pyrrole-2-carboxylic acid in 20% yield (Gottschalk, 1957*b*), evidently succeeds by initial formation of a Schiff base which makes the aldol condensation an intramolecular reaction. We soon replaced pyruvic acid by oxaloacetic acid in the expectation that reaction would be helped by a more reactive methylene group and that the superfluous carboxyl group could be eliminated even if it survived the aldol condensation. The new approach was successful, as already announced briefly (Cornforth, Daines & Gottschalk, 1957); we now present experimental details and evidence of the product's identity with *N*-acetylneuraminic acid of biological origin.

MATERIALS

N-Acetyl-D-glucosamine was prepared from D-glucosamine hydrochloride according to White (1940), and had m.p. 196° (decomp.); $[\alpha]_D^{18} + 75° \rightarrow +41°$ in water.

Oxaloacetic acid. Commercial ethyl sodio-oxaloacetate (100 g.; previously washed with ether) was added to a stirred mixture of 2N-H_2SO_4 (1 l.) and ether (250 ml.) at 0°. After 20 min. no solid remained; the aqueous layer was extracted with ether (3 × 100 ml.) and the combined ether solutions were washed with small quantities of ice-cold N-$NaHCO_3$ until the washings were no longer deeply coloured. The dried ($MgSO_4$) ether solution was evaporated and the residue distilled at 0·1 mm. to give ethyl oxaloacetate (58–65 g.), b.p. 66–70°. The ester was stored at 0° and hydrolysed as required by dissolving e.g. 30 g. in HCl (120 ml.; sp.gr. 1·18) and leaving for 2–3 days at room temperature. The crystalline oxaloacetic acid was collected, sucked as free as possible from liquid, and dried over KOH; this product (14·8 g.; m.p. 153–155°) was suitable for use. We find this method of preparation more reliable than that given by Heidelberger (1953).

Ion-exchange resins. Zeo-Karb 215 (40–100 mesh) was prepared (or regenerated) by washing with 2N-HCl until the effluent gave a negative flame test for Na^+ ion, then with water until the effluent gave no precipitate with $AgNO_3$. Dowex 1-X2 (50–100 mesh) was converted into the formate form by Svennerholm's (1956) procedure.

Activated carbon. Sutcliffe Speakman material grade 130 was boiled for 3 hr. with HNO_3 (sp.gr. 1·10), cooled, washed free of acid with water and passed, as aqueous suspension, through a 90-mesh sieve and on to a 280-mesh sieve. The material retained by the finer sieve was washed again with

dilute HNO_3 and with water, dried at 100° and stored. In packing the columns a few millimetres of Celite were first deposited and covered with filter paper; the carbon slurry was then poured on.

EXPERIMENTAL AND RESULTS

Preparation of synthetic N-*acetylneuraminic acid*

Oxaloacetic acid (4·5 g.) was dissolved in water (40 ml.) and brought to pH 9 by addition of 10 N-NaOH. *N*-Acetyl-D-glucosamine (10 g.) was added and the pH was adjusted potentiometrically to 11·0 by 5 N-NaOH. During the next 3 hr. frequent additions of alkali were needed to maintain this pH; thereafter, only occasional adjustment was required. After 17 hr. and 45 hr. samples of the mixture, submitted to the quantitative Bial assay (Werner & Odin, 1952), with *N*-acetylneuraminic acid as standard, gave values of 470 and 880 mg. respectively for the total content of *N*-acetylneuraminic acid in the mixture. The latter figure represents a yield of 8·5% based on the limiting reactant (oxaloacetic acid), but this is a maximal figure: other Bial-positive species are present and background absorption is uncertain. After 48 hr. the mixture was brought with acetic acid to pH 6·8 and preserved at 0° for several days before working up. Zeo-Karb 215 was added until evolution of CO_2 had ceased; the mixture was poured on a column of the same resin (35 mm. × 400 mm.), which was then washed with water until the effluent became neutral. The acidic effluent was added slowly to a Dowex 1-formate column (25 mm. × 230 mm.) which was afterwards washed with water until the effluent was free from substances giving a positive direct or indirect (pretreatment with 0·05 N-Na_2CO_3 at 100° for 3 min.) Ehrlich reaction. The acidic fraction was then eluted with 0·3 N-formic acid, 1250 ml. being required before the direct Ehrlich test on the effluent became weak. The effluent was concentrated almost to dryness in a rotary evaporator at 25°, redissolved in water and put on a column of activated carbon (40 mm. × 100 mm.) which was developed with water. The effluent was collected in 66 fractions of 22 ml. each. Fractions 9 to 48, which gave a strong direct Ehrlich reaction, were separately evaporated at about 30° *in vacuo*, yielding a total dry weight of about 840 mg. Spontaneous crystallization was noticed in fractions 13–26. Odd-numbered fractions 9–47 were chromatographed on paper (butanol–pyridine–water, 6:4:3, by vol.; descending). On spraying with orcinol–trichloroacetic acid three series of spots appeared, of R_F values 0·02, 0·08 and 0·13. Spots of R_F 0·02 appeared only in fractions 9 and 11. Spots of R_F 0·08 were observed with all fractions, and these spots coincided in R_F value and colour shade with the spot given by authentic *N*-acetylneuraminic acid. Spots of R_F 0·13 were seen with fractions 13–47, the intensity relative to the spot of R_F 0·08 increasing gradually until in the last fractions it was much the more intense.

Fractions 13–26 were separately crystallized by dissolution in a little water, evaporation *in vacuo* to small bulk and addition of acetic acid to a concentration of 60–70%. After crystallization had proceeded for 2 days at 5–10° the crystals were collected and washed with acetic acid and with ether. This product (172 mg.; $[\alpha]_D^{21} - 29°$ in water) was dissolved in water (0·5 ml.) and treated after filtration with acetic acid (2·5 ml.). After 3 days at 10° the white aggregated needles were collected (140 mg.); this product was solvated. A

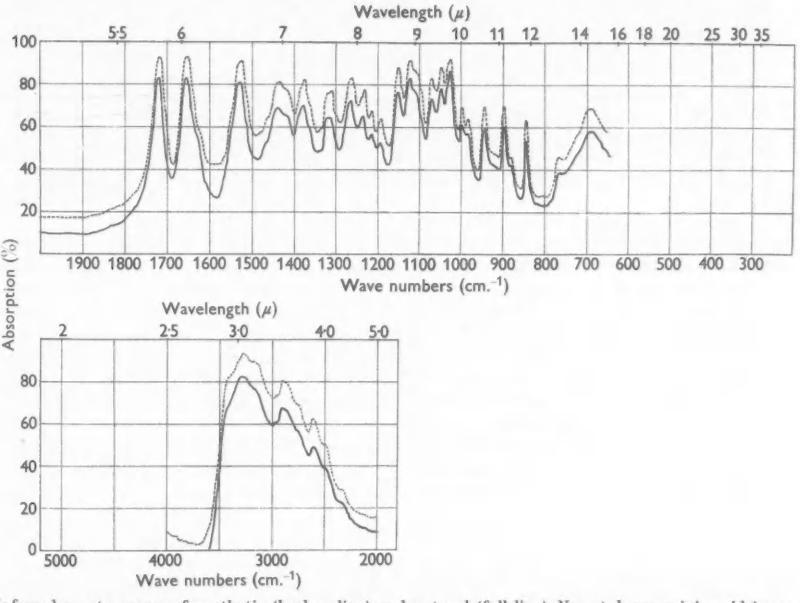


Fig. 1. Infrared spectrograms of synthetic (broken line) and natural (full line) *N*-acetylneuraminic acid in compressed potassium chloride. The curves for the natural product have been displaced slightly downward.

portion was crystallized from Blix's solvent system (Blix *et al.* 1956) with the proportions given by Kuhn & Brossmer (1956*b*): the acid (67 mg.) was dissolved in water (0·7 ml.), methanol (8 ml.) was added and then dry ether (18 ml.). A slight precipitate was removed by filtration through Celite and light petroleum (b.p. 40°–60°) was added until the clear filtrate became opalescent. Crystallization set in at once; after some hours at 20° it was completed at 0°. The product (42 mg.) was dried at 20° *in vacuo* over P_2O_5 and KOH.

Identification of synthetic N*-acetylneuraminic acid*

The product prepared as above formed snow-white partly aggregated needles. It appeared to be anhydrous, losing no weight on further drying at 56° in high vacuum, and was not hygroscopic (Found: C, 42·6; H, 6·2; N, 4·4%. Calc. for $C_{11}H_{19}O_9N$: C, 42·7; H, 6·2; N, 4·5%). It melted and decomposed at 185–187° in an evacuated capillary; in air it sometimes blackened without melting. Blix *et al.* (1956) gave m.p. 185–187°, Kuhn & Brossmer (1956*b*) gave m.p. 183–185°, for *N*-acetylneuraminic acid recrystallized in this manner. The product was laevorotatory, with $[\alpha]_D^{21}$ – 33° in water (*c*, 0·8); Blix *et al.* (1956), and Kuhn & Brossmer (1956*b*) gave $[\alpha]_D$ – 31°. The X-ray diffraction patterns of natural and synthetic specimens are compared in Pl. 1, and the infrared spectra in Fig. 1; the two specimens show no significant differences. In butanol–pyridine–water the two specimens ran as single spots of R_F 0·08.

DISCUSSION

From the data presented there can be no doubt that the synthetic material is chemically and stereochemically identical with *N*-acetylneuraminic acid as obtained from biological sources. The method of synthesis determines the length of the carbon chain in *N*-acetylneuramininic acid, and the nature and position of the functional groups. The presence of a pyranose ring follows from periodate oxidations (Blix *et al.* 1956).

Most of the configuration follows from the known stereochemistry of *N*-acetyl-D-glucosamine, but two details remain obscure: the stereochemistry at C-2 and at C-4. The mode of synthesis is of no assistance here, for several unproved assumptions are needed to deduce the configuration at C-4 from considerations of reaction mechanism; but such weight as can be accorded to negative evidence may be attached to the failure of *N*-acetylneuraminic acid to form lactones (Kuhn & Brossmer, 1956*b*), though it forms a methyl ester with exceptional ease. The pyranose ring in *N*-acetylneuraminic acid does not appear to be completely stable in solution, as is indicated by the ease of oxidation by hypoiodite (which presumably involves the α-oxo acid) and by the fact that periodate oxidation continues at an appreciable rate after the initial rapid consumption of 2 mol. prop. of reagent (Blix *et al.* 1956). Thus the configuration at C-2 must be regarded as mobile, even though mutarotation has not been observed; hence either of the two lactones, $1 \rightarrow 4$ or $1 \rightarrow 7$, might be formed from *N*-acetylneuraminic acid irrespective of configuration at C-4. Now since the acid is derived from *N*-acetyl-D-glucosamine, the trihydroxypropyl and acetamido substituents in the pyranose ring are *cis* to each other. If the hydroxyl group at C-4 is *cis* to these two groups it cannot take up the axial conformation necessary for lactonization without interference from the trihydroxypropyl group, which necessarily becomes axial at the same time. Contrariwise, the trihydroxypropyl group cannot become axial without interference from the hydroxyl group at C-4. This would provide a barrier to lactonization; a similar barrier would oppose pyranose formation from a γ-lactone formed from the open-chain tautomer of *N*-acetylneuraminic acid. The opposite configuration at C-4 presents no such obstacle to lactone formation by either route. Thus there is some indication that in *N*-acetylneuraminic acid the 4-hydroxyl group is *cis* to the trihydroxypropyl and acetamido groups.

The optimum conditions for condensation of *N*-acetyl-D-glucosamine with oxaloacetic acid have not yet been defined, but the yield of 1–2% obtained in these experiments can probably be improved.

Note added in proof. Kuhn & Brossmer (1957) have deduced from the optical rotation of a monocyclic mercaptal-lactone derived from *N*-acetylneuraminic acid that the hydroxyl group at C-4 is *trans* to the acetamido and trihydroxypropyl groups; and from the newly observed mutarotation of *N*-acetylneuraminic acid in dimethyl sulphoxide that the crystalline acid belongs to the β-series.

SUMMARY

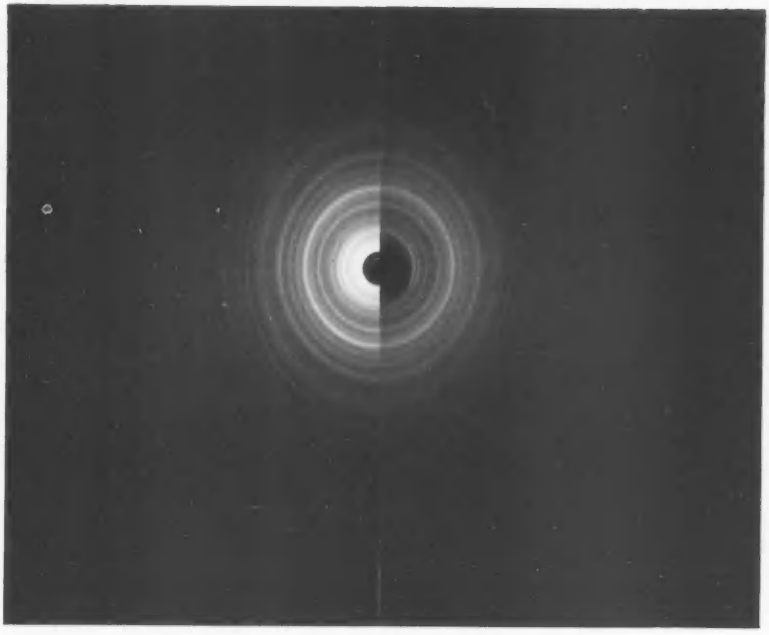
1. The occurrence of sialic acids in the animal organism is briefly reviewed.

2. The synthesis of crystalline *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine and oxaloacetic acid in aqueous solution at 20° and pH 11 is described.

3. The chemical and stereochemical identity of the synthetic product with *N*-acetylneuraminic acid of natural origin is shown by comparison of decomposition points, elementary analyses, optical rotations, infrared spectra and X-ray diffraction patterns.

4. The stereochemistry of *N*-acetylneuraminic acid is discussed.

We are grateful to Professor G. Blix, Uppsala, for a generous gift of authentic *N*-acetylneuraminic acid; to Dr R. K. Callow and Mr M. Dearden for the infrared spectra; to Mrs O. Kennard for the X-ray diffraction photographs; and to Mr J. Grant for willing help with the preparations.



X-ray diffraction patterns of synthetic *N*-acetylneuraminic acid (left half of photograph) and natural *N*-acetylneuraminic acid (right half). Some amorphous low-angle scattering is visible near the origin in the right half.

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(*Facing p.* 60)

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Studies in Detoxication

71. THE METABOLISM OF HYDROXYCOUMARINS*

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As a preliminary to the study of the fate of coumarin in animals (Mead, Smith & Williams, 1958), it was necessary to study the metabolic behaviour of the six isomeric hydroxycoumarins and to prepare either synthetically or biosynthetically their glucuronides and ethereal sulphates, since any of these compounds could be metabolites of coumarin.

The glucuronides of 3-, 4- and 7-hydroxycoumarin have been previously described (Flatow, 1910; Roseman, Heubner, Pancratz & Link, 1954; Mead, Smith & Williams, 1955), and Sieberg (1921) has reported that umbelliferone (7-hydroxy-), aesculetin (6:7-dihydroxy-) and daphnetin (7:8-dihydroxy-coumarin) after injection into rabbits were excreted in conjugated forms which were not described.

* Part 70: McIsaac & Williams (1957).

MATERIALS AND METHODS

Reference compounds. The following hydroxycoumarins were prepared: 3-, m.p. 153° (Linch, 1912), 4-, m.p. 206° (Boyd & Robertson, 1948), 5-, m.p. 228° (Adams & Bockstahler, 1952), 6-, m.p. 250–252° after recrystallization from dioxan (Bargellini & Monti, 1915) and 8-hydroxycoumarin, m.p. 159–160° (Cingolani, 1954).

Ethereal sulphates of hydroxycoumarins. Potassium salts of the hitherto undescribed ethereal sulphates of 3-, 5- and 8-hydroxycoumarins were synthesized by the general method of Burkhardt & Lapworth (1926) (see Table 1). The sulphate of 6-hydroxycoumarin was prepared in poor yield by the Elb's persulphate oxidation of coumarin. Coumarin (73 g.) and KOH (128 g.) were dissolved in 2 l. of water containing $FeSO_4,7H_2O$ (2 g.). Potassium persulphate (132 g.) was added over 8 hr. to the stirred solution kept below 20°. The solution was then brought to pH 2 with conc. H_2SO_4 and extracted twice with ether to remove coumarin. At this stage a large precipitate, mainly K_2SO_4,

Table 1. *Properties of the ethereal sulphates of the hydroxycoumarins**

The ethereal sulphate of 4-hydroxycoumarin could not be made by the method of Burkhardt & Lapworth (1926).

Potassium	Analysis found (%) K	S
2-Oxo-1:2-benzopyran-3-yl sulphate† (plates from aq. ethanol)	14·0	11·5
2-Oxo-1:2-benzopyran-5-yl sulphate† (needles from aq. 50% ethanol)	14·1	11·3
2-Oxo-1:2-benzopyran-6-yl sulphate‡ (plates from aq. 80% ethanol)	13·8	11·2
2-Oxo-1:2-benzopyran-8-yl sulphate† (prisms from water)	13·9	11·4
($C_9H_5O_6SK$ requires	13·9	11·4)

* Potassium 2-oxo-1:2-benzopyran-7-yl sulphate was described by Mead *et al.* (1955).
† Yields 80–85%, based on the weight of hydroxycoumarin taken.
‡ The light absorption of this compound in water was $\lambda\lambda_{max.}$ 273, 317 mμ with $\epsilon_{max.}$ 10 200 and 4800.

had formed. This was filtered off and extracted with 400 ml. of 66% (v/v) aq. acetone. Evaporation of the extract left a residue (3 g.) from which *potassium 2-oxo-1:2-benzopyran-6-yl sulphate* was obtained (see Table 1).

All these potassium salts were sparingly soluble in water and insoluble in ethanol; they did not melt below 300°. After a brief hydrolysis (1–5 min.) of these salts with boiling N-HCl the presence of the corresponding hydroxycoumarin could be demonstrated by paper chromatography. None of these sulphates in aqueous solution showed any fluorescence in u.v. light (360 mμ), but when exposed to u.v. light in N-NaOH they rapidly developed a blue–green or yellow–green fluorescence (photocatalytic effect of Feigl, Feigl & Goldstein, 1955).

The ethereal sulphates of 3-, 5-, 6-, 7- and 8-hydroxycoumarin and of 4-methyl-7-hydroxycoumarin were readily hydrolysed at pH 5·9 by an arylsulphatase preparation obtained from the gastric juice of Roman snails (*Helix pomatia*) (Jarrige & Henry, 1952).

Animals and dosing. Female chinchilla rabbits kept on a constant diet (see El Masry, Smith & Williams, 1956) were used and the coumarins were administered in aqueous suspension by stomach tube.

Analytical methods. Glucuronides in urine were determined by the naphtharesorcinol method as modified by Paul (1951). In this modification, 2 ml. of glucuronide solution containing 5–70 μg. of glucuronic acid was treated successively with 2 ml. of freshly prepared and filtered 0·35% (w/v) naphtharesorcinol in 0·1N-H_2SO_4, 3 ml. of 15N-H_2SO_4 and 0·2 ml. of 1% (w/v) aq. chloramine T, and the mixture heated in 6 in. × $\frac{5}{8}$ in. test tubes for 2 hr. in a boiling-water bath. The tubes were then cooled in ice for 10 min. and extracted with 8 ml. of ethyl acetate. The ethyl acetate layer was removed, adjusted to 10 ml., the optical density measured at 565 mμ and compared with values of a standard curve prepared under the same conditions with glucurone.

Ethereal sulphates were measured by the method of Sperber (1948).

Paper chromatography and colour reactions

Chromatography was carried out as previously described (Smith, Smithies & Williams, 1953). The R_F values, in various solvents, of the compounds concerned are given in Table 2. The following procedures were used for detecting the compounds on paper (see Table 2).

Photocatalytic fluorescence. The paper was sprayed with N-NaOH and exposed to u.v. light (360 mμ). Coumarin derivatives with no free hydroxyl groups and no substituent in the 4-position developed an intense fluorescence within 1 min. of exposure to u.v. light (Feigl *et al.* 1955).

Gibbs's reagent. A spray of 0·1% ethanolic 2:6-dichloroquinone chloroimide was followed by a spray of saturated $NaHCO_3$. Phenols in which the *para*-position to the hydroxyl is unsubstituted usually give blue colours with this reagent.

Brentamine fast blue B salt. Papers were sprayed first with a 0·01% aq. solution of this salt (stabilized, tetrazotized di-*o*-anisidine) and then with 2N-Na_2CO_3. Phenolic compounds give blue or purple colours.

Fluorescence. Some coumarin derivatives fluoresce under u.v. light (360 or 270 mμ), and a few quench the background fluorescence of the paper when illuminated with light of wavelength 270 mμ and showed up as dark spots on a light-purple background.

Isolation of metabolites

Optical rotations are given in Table 5.

3-Hydroxycoumarin. Urine from rabbits fed with 3-hydroxycoumarin gave a strong naphtharesorcinol reaction and reduced Benedict's solution slightly on boiling. It gave, with $FeCl_3$ solution, a weak green colour which was increased on boiling the urine with dilute acid. No precipitate was formed with 2:4-dinitrophenylhydrazine and no salicylic acid was found.

The 24 hr. urine of rabbits which had received a total of 4·2 g. of 3-hydroxycoumarin was treated with saturated normal lead acetate as described by Kamil, Smith & Williams (1951). The normal lead acetate precipitate was found to contain a glucuronide. This precipitate was suspended in water and the lead removed with H_2S. After separation of the lead sulphide, the solution deposited crystals (0·56 g.) of 3-hydroxycoumarin glucuronide (2-oxo-1:2-benzopyran-3-yl glucosiduronic acid). After recrystallization from water, it had m.p. 207–208° (Found: C, 51·4; H, 4·2; H_2O, 2·4. Calc. for $C_{15}H_{14}O_9,0{\cdot}5H_2O$, C, 51·8; H, 4·4; H_2O, 2·6%). Flatow (1910) records m.p. 207° and $[\alpha]_D - 72°$ in NaOH for this glucuronide. It did not reduce Benedict's reagent, and on hydrolysis with locust-crop liquor containing β-glucuronidase it yielded

Table 2. *R_F values and colour reactions of coumarin and its derivatives*

All runs were of 10 in. from the origin on Whatman no. 1 paper. Q means quenching of the background fluorescence of the paper when illuminated by light of wavelength 270 mμ and — indicates no colour reaction or fluorescence.

Compound	Solvent system*				Photocatalytic fluorescence	Gibb's reagent	Brentamine fast blue B salt	Colour of fluorescence excited by 360 mμ in NH_3 vapour
	A	*B*	*C*	*D*				
Coumarin	0·94	0·87	0·94	0·9	Blue–green	—	—	—
3-Hydroxycoumarin	0·85	0·50	0·27	0·8	—	Weak green	Blue	—
4-Hydroxycoumarin	0·90	0·63	0·15	0·21	—	Weak blue	Red	Weak blue
5-Hydroxycoumarin	0·85	0·72	0·58	0·17	—	Blue	Purple	Weak yellow
6-Hydroxycoumarin	0·82	0·70	0·84	0·06	—	—	Weak brown	Weak yellow
7-Hydroxycoumarin (umbelliferone)	0·88	0·52	0·42	0·14	—	—	—	Blue
8-Hydroxycoumarin	0·85	0·60	0·56	0·56	—	Blue	Purple	Q
6:7-Dihydroxycoumarin (aesculetin)	0·76	0·20	0·16	0·00	—	Weak brown	Brown	Weak blue-green
o-Coumaric acid	0·90	0·30	0·05	0·26	—	Blue	Purple	Blue–green
Salicylic acid	0·90	0·85	0·32	0·85	—	Blue	Orange	Blue
Melilotic acid	0·90	0·52	0·15	0·41	—	Blue	Purple	—
Potassium 3-hydroxycoumarin sulphate	0·52	0·78	0·66	0	Blue–green	—	—	—
Potassium 5-hydroxycoumarin sulphate	0·55	0·78	0·56	0	Blue–green	—	—	—
Potassium 6-hydroxycoumarin sulphate	0·47	0·70	0·46	0	Yellow–green	—	—	—
Potassium 7-hydroxycoumarin sulphate	0·50	0·75	0·73	0	Blue–green	—	—	—
Potassium 8-hydroxycoumarin sulphate	0·44	0·67	0·50	0	Yellow–green	—	—	—
3-Hydroxycoumarin glucuronide	0·56	0·13	0·03	0	Blue–green	—	—	—
4-Hydroxycoumarin glucuronide	0·58	0·30	0·04	0	—	—	—	Q
5-Hydroxycoumarin glucuronide	0·56	0·30	0·04	0	Blue–green	—	—	—
6-Hydroxycoumarin glucuronide	0·44	0·22	0·03	0	Yellow–green	—	—	—
7-Hydroxycoumarin glucuronide	0·45	0·27	0·03	0	Blue–green	—	—	Weak blue
8-Hydroxycoumarin glucuronide	0·48	0·15	0·03	0	Yellow–green	—	—	—
o-Coumaric acid glucuronide	0·62	0·05	0·00	0	—	—	—	Q
o-Hydroxy-*trans*-cinnamoyl glycine	0·86	0·45	0·03	0·12	—	Blue	Purple	Yellow–green

* Solvent *A* is *n*-butanol–acetic acid–water (4:1:5); *B* is propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88) (7:3); *C* is ethyl methyl ketone saturated with aq. 2N-NH_3 soln.; *D* is benzene–acetic acid–water (1:1:2) (all by vol.).

3-hydroxycoumarin, which was identified by its chromatographic behaviour on paper. Methylation with diazomethane, followed by acetylation with pyridine–acetic anhydride, yielded *methyl* (*2-oxo-1:2-benzopyran-3-yl tri-O-acetyl-β-D-glucosid*)*uronate* as colourless needles, m.p. 161–162°, from ethanol (Found: C, 54·9; H, 4·3. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6 %).

The basic lead acetate fraction of the urine was now prepared, and the lead-free filtrate was concentrated to 30 ml. On keeping this overnight, an ethereal sulphate crystallized out (0·3 g.). On hydrolysing this by boiling with 2N-HCl, free SO_4^{2-} ions and 3-hydroxycoumarin (m.p. and mixed m.p. 153°) were liberated.

4-Hydroxycoumarin. Urine from rabbits receiving 4-hydroxycoumarin gave a strong naphtharesorcinol reaction and reduced Benedict's reagent on prolonged boiling. No salicylic acid could be detected by paper chromatography in the urine before or after hydrolysis. The 24 hr. urine from rabbits which had received 9 g. of 4-hydroxycoumarin was fractionated by the lead acetate procedure, and a glucuronide was found in the basic lead acetate fraction. The lead was removed with H_2S and on concentration of this fraction to 100 ml. and keeping at 0° overnight the glucuronide crystallized (yield 1 g.). The mother liquor was concentrated *in vacuo* to a small volume and on careful addition of ethanol a further 0·5 g. of crystals was obtained. These crystals appeared to be a sparingly soluble salt of a glucuronide which was liberated from the salt (1·1 g.) by acidification with N-HCl. The glucuronide was identified as 2-oxo-1:2-benzopyran-4-yl glucosiduronic acid, which has previously been obtained by Roseman *et al.* (1954) from the urine of dogs given 4-hydroxycoumarin. The air-dried glucuronide appeared to be a monohydrate, m.p. 184–185° (Found: C, 51·1; H, 4·2; loss at 110° in 6 hr., 2·3. Calc. for $C_{15}H_{14}O_9,H_2O$, C, 50·6; H, 4·5; loss of $0·5H_2O$, 2·5 %), which on drying at 110° over P_2O_5 *in vacuo* left a hemihydrate (Found: C, 51·6; H, 4·1. Calc. for $C_{15}H_{14}O_9,0·5H_2O$, C, 51·8; H, 4·3 %). The glucuronide (an enol glycoside) reduced Benedict's solution on prolonged boiling. On hydrolysis with locust-crop liquor glucuronidase, or for a short time with 5N-HCl, 4-hydroxycoumarin, identified by paper chromatography, was liberated. (For the absorption spectrum of the glucuronide see the following paper, Mead *et al.* 1958).

Methyl(2-oxo-1:2-benzopyran-4-yl tri-*O*-acetyl-*β*-D-glucosid)uronate, prepared with diazomethane and pyridine–acetic anhydride, formed colourless needles of the *hemihydrate* from aq. ethanol, m.p. 124–125° (Found: C, 54·0; H, 4·4; OMe, 6·5; H_2O, 1·7. Calc. for $C_{22}H_{22}O_{12},0·5H_2O$, C, 54·0; H, 4·7; OMe, 6·4; H_2O, 1·9 %). Roseman *et al.* (1954) give m.p. 125–126° and $[\alpha]_D^{28} - 49·4°$ (*c*, 2 in $CHCl_3$) for this compound and describe it as a monohydrate.

5-Hydroxycoumarin. Urine from rabbits receiving 5-hydroxycoumarin gave a strong naphtharesorcinol reaction and did not reduce Benedict's solution. Some unchanged 5-hydroxycoumarin, together with its ethereal sulphate and glucuronide, were detected on paper chromatograms of the urine. No other metabolites were detected. The 24 hr. urine from rabbits which had received 5·7 g. of 5-hydroxycoumarin (850 ml.) was brought to pH 4–5 with acetic acid and 100 ml. of saturated lead acetate soln. was added. The amorphous precipitate was rapidly removed by centrifuging and the supernatant liquid allowed to stand at 0° for 0·5 hr., when a lead salt of the glucuronide deposited as a mass of woolly needles. These were filtered off, suspended in 750 ml. of water at 70–80° and the solution was saturated with H_2S. After removal of PbS, the solution was cooled to 0° and the crystals (2·4 g.) of *2-oxo-1:2-benzopyran-5-yl β-D-glucosiduronic acid* were collected and recrystallized from water. It formed sheaves of needles, m.p. 202–203° (decomp.) (Found: C, 48·1; H, 4·8. $C_{15}H_{14}O_9,2H_2O$ requires C, 48·1; H, 4·8 %). It lost water on drying at 110° but the anhydrous compound was hygroscopic and rapidly regained the lost weight when exposed to air. Hydrolysis of the glucuronide with snail gastric-juice glucuronidase or with 5N-HCl liberated 5-hydroxycoumarin, which was identified by paper chromatography.

The triacetyl methyl ester of 5-hydroxycoumarin glucuronide could not be prepared successfully by methylation with diazomethane followed by acetylation with pyridine–acetic anhydride as described by Kamil *et al.* (1951). The glucuronide had to be acetylated first with acetic anhydride, with perchloric acid as catalyst, and then methylated with methyl iodide and silver oxide. The glucuronide (100 mg.) was dissolved in acetic anhydride (0·4 ml.) and one drop of 60 % aq. $HClO_4$ added. The reaction was immediate, with evolution of heat, and in 2 min. the triacetyl glucuronide separated. A few drops of water were added to decompose excess of acetic anhydride and then an equal volume of ethanol. The triacetyl acid (80 mg.) on recrystallization from water formed needles, m.p. 220–221° (decomp.). The dry acid was dissolved in 50 ml. of $CHCl_3$ and methyl iodide (1 ml.) and silver oxide (3 g.) were added. The mixture was kept for 1 hr., then filtered and concentrated to a glassy solid. The latter was crystallized from ethanol to give needles (70 mg.) of *methyl* (*2-oxo-1:2-benzopyran-5-yl tri-O-acetyl-β-D-glucosid*)*uronate*, m.p. 158–159° (Found: C, 55·5; H, 4·9. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6 %).

6-Hydroxycoumarin. A total of 8 g. of 6-hydroxycoumarin was fed to four rabbits. The 24 hr. urine gave an intense naphtharesorcinol reaction and did

not reduce Benedict's solution. When acidified the urine showed a dull blue–green fluorescence in u.v. light (360 mμ) and when made alkaline with a few drops of aq. NH_3 soln. the fluorescence was an intense blue. Paper chromatography of the urine showed the presence of some 6-hydroxycoumarin and its ethereal sulphate, and a substance fluorescing a bright blue. The latter substance was probably a 6-conjugated aesculetin (6:7-dihydroxycoumarin), since the blue fluorescence disappeared on hydrolysing the urine, and aesculetin as well as 6-hydroxycoumarin was detected on paper chromatograms of the acid-hydrolysed urine.

The glucuronide fraction of the urine, obtained from the basic lead acetate precipitate in the usual way, was reduced to 50 ml. *in vacuo* and treated with 200 ml. of ethanol, and a gummy precipitate was formed and separated. The supernatant liquid was separated from the precipitate, concentrated to 50 ml. at 40° *in vacuo* and neutralized with solid $KHCO_3$, when plates of the potassium salt of the ethereal sulphate of 6-hydroxycoumarin were deposited (0·31 g.) and were recrystallized from water (Found: K, 13·9; S, 11·4. $C_9H_5O_6SK$ requires K, 13·9; S, 11·4%). Hydrolysis of the salt with 2N-HCl gave a crystalline precipitate of 6-hydroxycoumarin, m.p. and mixed m.p. 225–226°. The u.v. spectrum ($\lambda_{max.}$ 273, 317 mμ; $\epsilon_{max.}$ 10 200, 4800 in water) and chromatographic behaviour of this sulphate were identical with a synthetic sample.

The above alcohol-insoluble gum was redissolved in a little water and treated with excess of ethanol, and a glucuronide separated which was eventually obtained as a dry white powder (3·5 g.). On recrystallization of the powder from a small amount of water, the ammonium salt of 6-hydroxycoumarin glucuronide was obtained as fine needles. The *ammonium (2-oxo-1:2-benzopyran-6-yl β-D-glucosid)uronate* decomposed at 204–205° (Found: C, 49·3; H, 4·5; N, 3·8. $C_{15}H_{17}O_9N,0{\cdot}5H_2O$ requires C, 49·45; H, 5·0; N, 3·8%). The NH_4^+ ion was detected by Nessler's reagent, and the salt yielded 6-hydroxycoumarin (detected chromatographically) when incubated with β-glucuronidase (snail gastric juice) at pH 4·6. The free glucosiduronic acid was not readily obtainable in a crystalline form, and the NH_4^+ ion present in the salt isolated was probably derived from the aq. NH_3 soln. usually added to the urine when preparing the glucuronide fraction with basic lead acetate. *Methyl (2-oxo-1:2-benzopyran-6-yl tri-O-acetyl-β-D-glucosid)uronate*, m.p. 174–175°, was prepared from the above salt with acetic anhydride and perchloric acid, followed by methyl iodide and silver oxide as described for the preparation of the 5-isomer (Found: C, 55·2; H, 4·4. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6%).

8-Hydroxycoumarin. Urine from rabbits receiving 0·5 g. of 8-hydroxycoumarin/kg. gave a strong naphtharesorcinol reaction and no Benedict's or $FeCl_3$ reactions. Paper chromatography of urine showed the presence of spots corresponding with 8-hydroxycoumarin, its ethereal sulphate and its glucuronide.

The basic lead acetate fraction of the 24 hr. urine of five rabbits which had received a total of 7·5 g. of 8-hydroxycoumarin was concentrated to 300 ml. and the glucuronide precipitated with excess of ethanol. The precipitate (6·3 g.) was dissolved in water (100 ml.) and the solution acidified with conc. HCl. On cooling to 0°, fine colourless needles were deposited (4·65 g.). After recrystallization from water the *2-oxo-1:2-benzopyran-8-yl β-D-glucosiduronic acid* had m.p. 158–160° (decomp.) (Found: C, 50·5; H, 4·3; H_2O, 5·2. $C_{15}H_{14}O_9,H_2O$ requires C, 50·6; H, 4·5; H_2O, 5·1%). Hydrolysis with 5N-HCl or β-glucuronidase gave 8-hydroxycoumarin, which was identified on paper chromatograms. *Methyl (2-oxo-1:2-benzopyran-8-yl tri-O-acetyl-β-D-glucosid)uronate* (m.p. 135–136°) was prepared with diazomethane and pyridine–acetic anhydride (Found: C, 54·2; H, 5·1; H_2O, 1·6. $C_{22}H_{22}O_{12},0{\cdot}5H_2O$ requires C, 54·2; H, 4·7; H_2O, 1·9%).

The ethanolic solution remaining from the precipitation of the crude glucuronide salt was concentrated to 50 ml. *in vacuo* and neutralized with $KHCO_3$, when lustrous plates of the potassium salt of the ethereal sulphate of 8-hydroxycoumarin were deposited and shown to be chromatographically identical with the synthetic material (Found: K, 13·8; S, 11·2. $C_9H_5O_6SK$ requires K, 13·9; S, 11·4%). A short acid hydrolysis yielded 8-hydroxycoumarin, m.p. and mixed m.p. 159–160°.

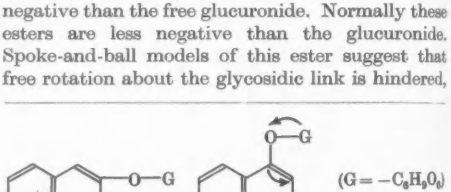
RESULTS AND DISCUSSION

The conjugations of the hydroxycoumarins are shown in Table 3, which also includes the results obtained with umbelliferone and 4-methyl-umbelliferone (Mead *et al.* 1955). The figures for the glucuronic acid conjugation of 5-, 6-, 7- and 8-hydroxycoumarins are probably low, since it was found that the glucuronides of these coumarins are relatively stable to acid and are not completely hydrolysed by the acid used in the Paul (1951) modification of the naphtharesorcinol method. Some results with pure aqueous solutions of glucuronides in the Paul method are given in Table 4. Glucuronides relatively stable to acid and giving low results with the naphtharesorcinol method have been mentioned previously by Hanson, Mills & Williams (1944).

3-, 5-, 6-, 7- and 8-Hydroxycoumarins are phenolic in nature and are metabolized in rabbits mainly by direct conjugation with glucuronic and sulphuric acids. 4-Hydroxycoumarin (benzotetronic acid) differs from its isomers in that it

conjugates only with glucuronic acid. It formed no ethereal sulphate *in vivo* and it was the only hydroxycoumarin from which no ethereal sulphate could be obtained chemically. 4-Hydroxycoumarin is a relatively strong acid of pK 5·8, and it would not be expected to form an ethereal sulphate when fed to the intact animal. This is apparently in agreement with the suggestions of Anderton, Smith & Williams (1948) that hydroxy compounds with pK_a values less than about 7 do not form ethereal sulphates *in vivo*. The reason for this may be either that the compound does not penetrate to the site of sulphate synthesis or, if an ethereal sulphate is formed, that it is too unstable to persist. The glucuronide of 4-hydroxycoumarin also differed from the glucuronides of the other hydroxycoumarins, in that it reduced hot Benedict's reagent whereas the others did not. Both 3- and 4-hydroxycoumarin glucuronides are enol-glycosides and the reducing properties of the 4-glucuronide (II) can be attributed to the weakening of the aglycone–glucuronic acid link by the inductive effect of the carbonyl oxygen transmitted through the 3:4 double bond of the coumarin nucleus. 3-Hydroxycoumarin glucuronide (I) is non-reducing since such electron shifts cannot readily occur.

The specific and molecular rotations of the isomeric hydroxycoumarin glucosiduronic acids and their triacetyl methyl esters are shown in Table 5. One abnormality requires comment and that is that the rotation of the triacetyl methyl ester of 8-hydroxycoumarin β-glucosiduronic acid is more negative than the free glucuronide. Normally these esters are less negative than the glucuronide. Spoke-and-ball models of this ester suggest that free rotation about the glycosidic link is hindered,



(I) (II)

Table 3. *Conjugation of hydroxycoumarins in the rabbit* (*dose* 0·2 *g./kg.*)

Compound	Conjugation as percentage of dose*		
	Glucuronide	Ethereal sulphate	Total
3-Hydroxycoumarin	57 (55–59)	14 (12–19)	71
4-Hydroxycoumarin	37 (31–44)	1† (0–3)	37
5-Hydroxycoumarin	37‡ (28–45)	18 (15–20)	55‡
6-Hydroxycoumarin	48‡ (46–53)	28 (23–35)	76‡
7-Hydroxycoumarin	41‡ (39–42)	21 (19–22)	62‡
4-Methyl-7-hydroxycoumarin	49 (45–53)[5]	4 (2–7)[5]	53
8-Hydroxycoumarin	60‡ (50–71)	8 (4–12)	68‡

* Three animals were used unless otherwise indicated by superior figures outside parentheses.
† This figure is not significant.
‡ These figures are probably low owing to the stability of the glucuronides (see Table 4).

Table 4. *Recovery of glucuronides by the naphtharesorcinol method* as modified by Paul* (1951)

Glucuronide	Recovery (%)	Glucuronide of	Recovery (%)	Glucuronide	Recovery (%)
p-Chlorophenyl	99	3-Hydroxycoumarin	86	(−)Menthyl	107
p-Bromophenyl	99	4-Hydroxycoumarin	79	(+)Menthyl	99
p-Iodophenyl	104	5-Hydroxycoumarin	63	(+)*iso*Menthyl	91
p-Aminophenyl	92	6-Hydroxycoumarin	67	(+)*neo*Menthyl	112
p-Cyanophenyl	91	7-Hydroxycoumarin	47	2-Ethylhexanoyl	106
o-Amino-*p*-sulphonamidophenyl	39	4-Methyl-7-hydroxycoumarin	79	Di-*iso*propylmethyl	98

* See text for method.

Table 5. *Optical rotations of the hydroxycoumarin β-glucosiduronic acids*

Isomer	Hydroxycoumarin glucuronide		Triacetyl methyl esters	
	$[\alpha]_D$*	$[M]_D/100$	$[\alpha]_D$* (*c*, 1 in $CHCl_3$)	$[M]_D/100$
3	−98° (*c*, 1 in N-NaOH)	−340°	−78°	−373°
4	−85° (*c*, 1 in ethanol)	−303	−50·5	−246
5	−61° (*c*, 0·25 in water)	−228	−50	−239
6	−86° (NH_4 salt, *c*, 1 in water)	−313	−28·5	−136
7	−105° (*c*, 1 in water)	−374	−44	−210
8	−77° (*c*, 1 in water)	−274	−136	−650

* t = 20–25°.

because of the space relation of the triacetyl glucuronic acid methyl ester portion of the molecule to the coumarin ring (cf. Kamil *et al.* 1951).

SUMMARY

1. The fate of 3-, 4-, 5-, 6- and 8-hydroxycoumarins in the rabbit has been studied. All five compounds are conjugated with glucuronic acid and the glucuronides have been isolated and described. With the exception of 4-hydroxycoumarin, they are also excreted in conjugation with sulphuric acid, and the ethereal sulphates of 3-, 6- and 8-hydroxycoumarins were isolated from the urine. Hydroxylation of 6-hydroxycoumarin to 6:7-dihydroxycoumarin (aesculetin) was also observed.

2. The potassium salts of the sulphuric esters of 3-, 5-, 6- and 8-hydroxycoumarins have been synthesized and described.

3. The paper-chromatographic behaviour and the colour reactions of the hydroxycoumarins and their conjugates have been described.

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Studies in Detoxication

72. THE METABOLISM OF COUMARIN AND OF *o*-COUMARIC ACID

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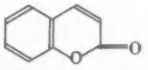
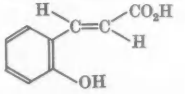
Coumarin (2-oxo-1:2-benzopyran) is the sweet-smelling constituent of white clover, and it has been found in a large variety of plants. Many of its derivatives are pharmacologically active and they include anticoagulant drugs (see Hunter & Shepherd, 1955), rodenticides and insecticides. Coumarin itself has been employed for flavouring food, but its use has been discouraged owing to its damaging effect upon the liver in animals.

No previous study on the metabolic fate of coumarin has been made, except that Vasiliu, Timosencu, Zaimov & Coteleu (1938) fed it to sheep and found that it did not produce benzoic acid derivatives. However, all naturally occurring derivatives of coumarin, except dicoumarol, are either 7-hydroxy derivatives or 7-*O*-ethers of coumarin (see tables in Elderfield, 1951). This suggests that the 7-position is a possible point of biological attack on the coumarin molecule. In fact, it has already been shown that in man ethyl biscoumacetate (Tromexan) is hydroxylated in the 7-position of one of its coumarin rings (Burns, Weiner, Simson & Brodie, 1953; Burns, Wexler & Brodie, 1953).

Coumarin (I) is the lactone of *o*-hydroxy-*cis*-cinnamic acid (coumarinic acid). It was therefore



(I)



(II)

of interest to know something about the metabolism of the *trans*-isomer, *o*-coumaric acid (*o*-hydroxy-*trans*-cinnamic acid, II), since this acid could be a metabolite of coumarin.

A study of the metabolism of the hydroxycoumarins has been described in the preceding paper, which contains data relevant to this work (Mead, Smith & Williams, 1958).

EXPERIMENTAL

Animals. For most of the quantitative data and the isolation of metabolites, chinchilla rabbits kept on a constant diet were used. Other species are referred to in the text.

Reference compounds. Coumarin, m.p. 69–70°, and *o*-coumaric acid monohydrate, m.p. 208–209°, were purchased and purified. *o*-Methoxy-*trans*-cinnamic acid, m.p. 185–186°, was prepared by methylation of *o*-coumaric acid with dimethyl sulphate and NaOH in the usual manner, and melilotic acid (*β*-*o*-hydroxyphenylpropionic acid), m.p. 83–84°, by the action of alkali on dihydrocoumarin (Light and Co.).

Acetyl *o*-coumaryl chloride, m.p. 54° (5 g., Houben & Pfankuch, 1926) and an excess of glycine (10 g.) were shaken with aq. 10% (w/v) Na_2CO_3 soln. (100 ml.) until the chloride had dissolved. The yellow solution was treated with charcoal, filtered and acidified with conc. HCl. A pasty precipitate separated, which was triturated in water until it solidified and then recrystallized from a large volume of hot water. This crude acetyl coumaryl glycine was dissolved in a slight excess of 2N-NaOH and the solution boiled for 5 min. After cooling, and acidifying the solution with conc. HCl, *o-hydroxy*-trans-*cinnamoylglycine* (1 g.) was obtained as colourless prisms, m.p. 226–227° after recrystallization from methanol (Found: C, 59·6; H, 5·0; N, 6·0. $C_{11}H_{11}O_4N$ requires C, 59·7; H, 5·0, N, 6·3%).

Analytical methods. Glucuronic acid and ethereal sulphates were determined as in the preceding paper (Mead *et al.* 1958). Absorption spectra were measured with a Unicam SP. 500 spectrophotometer.

Umbelliferone (7-hydroxycoumarin) in urine was determined fluorimetrically (with an H 553 Spekker fluorimeter) as described by Mead, Smith & Williams (1955).

To determine conjugated umbelliferone in urine, the conditions of hydrolysis of the ethereal sulphate and glucuronide of umbelliferone had first to be investigated. Potassium umbelliferone sulphate added to urine was completely hydrolysed by heating on the water bath for 30 min. with 0·1N-HCl, 1 ml. of urine and 2 ml. of 0·15N-HCl being used. The recovery of umbelliferone, estimated fluorimetrically, was quantitative. Under these conditions only 2% of the glucuronide is hydrolysed. With umbelliferone glucuronide stronger acid and longer heating were necessary. When 1 ml. of urine containing about 0·5 mg. of the glucuronide was heated under reflux for 3 hr. with 2 ml. of 9N-HCl, the recovery of umbelliferone was 80±2%. When umbelliferone was added to normal urine and the solution subjected to the above hydrolysis conditions, it could be recovered quantitatively. The estimation of conjugated umbelliferone in urine was therefore carried out as follows. The urine (1 ml.) was mixed with 0·15N-HCl (2 ml.) and heated on the water bath for 30 min. After cooling, 2 ml. of 0·1N-NaOH was added and the solution was made up to 100 ml. with 0·1M-glycine buffer, pH 10·3. The fluorescence of this solution was measured as such or after further dilution with buffer if necessary. This gave an estimate of umbelliferone conjugated as sulphate. For the estimation of total umbelliferone, 1 ml. of urine and 2 ml. of 9N-HCl were heated under reflux for 3 hr. The solution was neutralized with 3 ml. of 5N-NaOH and diluted to 100 ml. with water. Then 1 ml. of this solution was diluted to 25 ml. with glycine buffer and the fluorescence measured. In calculating the umbelliferone conjugated with glucuronic acid, a correction was made to allow for the fact that only 80% of the glucuronide is hydrolysed under these conditions.

Isolation of metabolites from coumarin

Rabbits were given, by stomach tube, doses of 0·1 g. of coumarin suspended in water/kg. Doses higher than this were too toxic and many of the animals died when given doses of 0·2 g./kg. Preliminary studies on the urine after coumarin dosing showed that it was non-reducing and gave a strong naphtharesorcinol reaction. When acidified, the urine showed a slight blue–green fluorescence in u.v. light, but on neutralizing or adding aq. NH_3 soln. the fluorescence became an intense blue indicating the presence of umbelliferone. Paper chromatography of the urine before and after acid hydrolysis indicated the presence of 3-, 7- and a trace of 8-hydroxycoumarins. *o*-Coumaric acid was not identified, and there were no indications of the presence of 4-, 5- or 6-hydroxycoumarins.

A total of 27 g. of coumarin was fed to several rabbits at the rate of 0·5 g./rabbit/day. The urine (5 l.) and faeces (320 g.) were collected for 5 days. The urine was worked up by systematic lead acetate precipitation as described by Kamil, Smith & Williams (1951). The precipitate from normal lead acetate contained no appreciable quantities of metabolites.

Isolation of 3-hydroxycoumarin conjugates. The basic lead acetate precipitate was freed of lead and the filtrate concentrated *in vacuo* at 40–45° to 200 ml. After keeping overnight at 0°, two kinds of crystals separated: small plates (*A*) filling the bulk of the solution and small needles (*B*) adhering to the bottom of the flask. The crystals were separated by decantation.

The crystals *A* (0·78 g.) were neutral in reaction and gave a strong test for ethereal sulphate and potassium. They were recrystallized from 80% ethanol and formed plates which did not melt on heating to 360°. They were identified as potassium 2-oxo-1:2-benzopyran-3-yl sulphate (Found: K, 14·1; S, 11·3. Calc. for $C_9H_5O_6SK$, K, 13·9; S, 11·4%). On hydrolysis with 0·3N-HCl they yielded 3-hydroxycoumarin, m.p. and mixed m.p. 153°. On paper chromatograms they behaved identically with the ethereal sulphate of 3-hydroxycoumarin (Mead *et al.* 1958).

The crystals *B* (0·46 g.) were acid in reaction, non-reducing, and gave a strong naphtharesorcinol reaction, but no ethereal sulphate test. They were recrystallized from water as colourless needles, m.p. 207–208° (decomp.) and $[\alpha]_D^{20} - 98{\cdot}5°$ in N-NaOH (*c*, 1). They were identified as 2-oxo-1:2-benzopyran-3-yl β-D-glucosiduronic acid (mixed m.p. 207–208°, decomp.) and their chromatographic behaviour was identical with that of authentic samples of this glucuronide (Mead *et al.* 1958). Concentration of the mother liquor from *A* and *B* gave a further yield (1·06 g.) of this glucuronide (m.p. 207–208° and $[\alpha]_D^{20} - 98°$ in N-NaOH).

Isolation of 7-hydroxycoumarin. The filtrates (50 ml.) from the isolation of the 3-hydroxycoumarin conjugates were now treated with acetone (500 ml.), and on keeping overnight a gummy precipitate separated. The acetone was decanted from the precipitate, which was acid, non-reducing and gave a strong naphtharesorcinol reaction and a slight ethereal sulphate test. No crystalline product could be isolated from the precipitate. It was therefore dissolved in 5N-HCl, heated under reflux for 3 hr., cooled and extracted continuously with ether for 24 hr. The ether extract was then chromatographed on a cellulose-powder column (Hough, 1954), ethyl methyl ketone saturated with aq. 2N-NH_3 soln. being used as solvent. Umbelliferone was known to be present in this fraction and could be followed on the column by its fluorescence in u.v. light. The main fluorescent band on the column was due to this compound, although there were other minor fluorescent bands which on elution gave only minute quantities of solids. The umbelliferone eluate was concentrated to yield 157 mg. of 7-hydroxycoumarin, m.p. and mixed m.p. 230–231° after recrystallization from water (charcoal). The acetone decanted as above was evaporated to yield a gum, which on similar treatment yielded 23 mg. of 7-hydroxycoumarin.

The filtrate (5 l.) from the basic lead acetate precipitate was concentrated *in vacuo* to 500 ml. and treated with ethanol, and the bulky inorganic precipitate was filtered off. The filtrate was evaporated to a syrup which was hydrolysed, extracted with ether and chromatographed on a cellulose column as described in the preceding paragraph. This fraction yielded a further 210 mg. of 7-hydroxycoumarin (total yield, 390 mg.). The 7-hydroxycoumarin (350 mg.) was acetylated with acetic anhydride and anhydrous sodium acetate and yielded 370 mg. of 7-acetoxycoumarin, m.p. and mixed m.p. 140–141° after recrystallization from hot water (charcoal) (Found: umbelliferone, 77·1 %, determined fluorimetrically. Calc. for $C_{11}H_8O_4$, umbelliferone, 79·4 %).

Examination of the faeces. The dried faeces were extracted with ether in a Soxhlet extractor. Paper chromatography of the extract revealed no coumarin or hydroxycoumarin.

Examination of tissues. A rabbit which had received 170 mg. of coumarin/kg. died 12 hr. later. The gastric contents appeared to contain no coumarin, by the sensitive test of Feigl, Feigl & Goldstein (1955). It was, however, detected readily in the visceral fat.

Hydroxylation of coumarin in various species. Coumarin was administered to five species (see Table 3) and the 24 hr. urine collected. It was made 5N with respect to HCl by the addition of conc. HCl and then heated under reflux for 3 hr. After cooling, the hydrolysate was continuously extracted with ether for 6 hr. The extract was evaporated *in vacuo* to a gum and a small portion of this gum was chromatographed on Whatman no. 1 paper. It was placed at the origin with a platinum loop and then irrigated with the solvents (see preceding paper). The metabolites were detected as previously described (Mead *et al.* 1958) and the findings are summarized in Table 3.

Isolation of metabolites from o-coumaric acid

o-Coumaric acid (1 g./rabbit) was administered by stomach tube dissolved in aq. $NaHCO_3$ soln. The urine was slightly reducing towards Benedict's reagent and gave a strong naphtharesorcinol test. The urine as such (one drop) when chromatographed on paper in solvents *A* and *B* (Mead *et al.* 1958) appeared to contain *o*-coumaric acid, 4- and 7-hydroxycoumarins, *o*-hydroxy-*trans*-cinnamoylglycine and a substance which quenched the background fluorescence of the paper in u.v. light. This last substance appeared to be the glucuronide of *o*-coumaric acid. After hydrolysis (0·5 ml. of urine + 0·5 ml. of conc. HCl heated for 5 min.) chromatography showed increased amounts of *o*-coumaric acid, and of 4- and 7-hydroxycoumarins. The *o*-hydroxy-*trans*-cinnamoylglycine spot and the quenching spot had disappeared. Several other spots were detected on the paper by various reagents, but were not identified. 3-, 5-, 6- and 8-Hydroxycoumarins, melilotic acid and salicylic acid were not detected in either the unhydrolysed or hydrolysed urines.

Melilotic acid (*o*-hydroxyphenylpropionic acid) also was fed to rabbits and the urine examined chromatographically. The 24 hr. urine of a rabbit which had received an oral dose of 1 g. of the acid was examined as above for *o*-coumaric acid. The urine gave a strong naphtharesorcinol reaction, was slightly reducing but gave no colour reaction with $FeCl_3$ soln. It fluoresced in u.v. light, a pale blue–green in acid and neutral conditions and an intense yellow–green when treated with NH_3 soln. Ether extracts of the acidified urine were chromatographed and spots corresponding to melilotic acid,

o-coumaric acid, *o*-hydroxy-*trans*-cinnamoylglycine and 4- and 7-hydroxycoumarins were found.

Isolation of 4-hydroxycoumarin glucuronide. A total of 6 g. of *o*-coumaric acid (as sodium salt) was fed to six rabbits. The 24 hr. urine (760 ml.) gave a strong naphtharesorcinol reaction and slightly reduced Benedict's reagent. On making strongly alkaline with aq. NH_3 soln. the urine fluoresced an intense greenish yellow owing to the presence of free *o*-coumaric acid. The glucuronide fraction of the urine was separated through basic lead acetate and the lead-free filtrate was reduced *in vacuo* to 50 ml. This solution was strongly reducing to Benedict's reagent. Excess of ethanol was added and a precipitate of inorganic material was discarded. The solution (200 ml.) was again reduced *in vacuo* to 50 ml., treated with acetone (350 ml.) and kept overnight at 0°. The acetone was decanted from the gum which had formed. This gum was now taken up in ethanol and the solution concentrated *in vacuo* to 10 ml. On keeping at 0° crystals (30 mg.) of a glucuronide separated. These were filtered off and the filtrate was examined later (see below). The crystals reduced hot Benedict's reagent and contained some inorganic material. They were therefore dissolved in 2 ml. of water and a few drops of 2N-HCl added. On keeping at 0° the glucuronide separated as long needles. Further recrystallization yielded 9 mg. of crystals, which were identified as 2-oxo-1:2-benzopyran-4-yl glucosiduronic acid, m.p. and mixed m.p. 184–185°. Its u.v. spectrum in ethanol was identical with that of the authentic glucuronide (see Table 1). On hydrolysis with 5N-HCl or locust-crop liquor containing β-glucuronidase at pH 4·6 it yielded 4-hydroxycoumarin, which was identified chromatographically in two solvents (see Mead *et al.* 1958).

Isolation of o-glucosidurono-trans-cinnamic acid. The filtrate from the separation of 4-hydroxycoumarin glucuronide described in the preceding paragraph was acidified further with a few drops of 2N-HCl, treated with charcoal and filtered. On keeping overnight at 0° a glucuronide (66 mg.) (naphtharesorcinol test) separated with m.p. 228–229° (decomp.) after recrystallization from water (charcoal). It was non-reducing and contained no free phenolic groups, but was optically active, $[\alpha]_D^{18} - 81{\cdot}3°$ in 0·1N-NaOH (*c*, 0·8). It analysed as the *hemihydrate* of o-β-D-*glucosidurono*-trans-*cinnamic acid* (Found: C, 51·6; H, 4·8. $C_{15}H_{16}O_9, 0{\cdot}5H_2O$ requires C, 51·6; H, 4·9%). The water was not lost on heating at 110°. It titrated with 0·02N-NaOH as a dibasic acid, the equivalent weight found being 174·6, which is half the required mol.wt. 349·3. On hydrolysis with 5N-HCl or with locust-crop liquor containing β-glucuronidase it yielded *o*-coumaric acid, which was identified by its R_F value in four solvents (see Mead *et al.* 1958) and its characteristic blue–green fluorescence at alkaline pH values in u.v. light. Its u.v. absorption spectrum would be expected to be similar to that of *o*-methoxy-*trans*-cinnamic acid. The spectra of these two compounds together with those of coumarin, *o*-coumaric acid and *o*-hydroxy-*trans*-cinnamoylglycine are given in Table 1. The major band in all four compounds is at 274 mμ, and the $\epsilon_{max.}$ of the glucuronide has a *trans* value (i.e. about 18 000), whereas $\epsilon_{max.}$ for coumarin, a *cis*-derivative, is 11 000. The methyl ether of coumaric acid, however, shows a peak at 225 mμ, and this appears also in the glucuronide, which is similarly an ether. This supports the view that the compound is an ether glucuronide of *o*-coumaric acid.

Isolation of o-coumaric acid. This compound sometimes separated from the lead-free filtrate of the glucuronide fraction of the urine. In one experiment in which 12 g. of *o*-coumaric acid had been fed, 1 g. of the free acid, m.p. 208–209°, was isolated by continuous ether extraction of the acidified urine for 24 hr.

Action of ascorbic acid and Fenton's reagent on coumarin

Udenfriend, Clark, Axelrod & Brodie (1954) have shown that the pattern of oxidation of a number of organic compounds by a system containing ascorbic acid, Fe^{2+} ions and ethylenediaminetetra-acetic acid was similar to that found *in vivo*. Boyland & Sims (1953), using Fenton's reagent, which is regarded as a source of hydroxyl-free radicals, showed that coumarin was oxidized to 7-hydroxycoumarin. Since all the monohydroxycoumarins

Table 1. *Ultraviolet-absorption spectra in ethanol of coumarin, o-coumaric acid and some of their derivatives*

	Wavelength of absorption peaks ($\lambda_{max.}$) (mμ)			Corresponding molecular-extinction coefficients ($10^{-3}\ \epsilon_{max.}$)		
Coumarin	—	274	310	—	11·1	5·7
4-Hydroxycoumarin	—	268, 278	304	—	10·7, 11·6	8·5
4-Glucosiduronocoumarin	—	268, 278	304	—	10·6, 10·2	6·6
o-Hydroxy-*trans*-cinnamic acid	—	274	325–326	—	18·3	10·1
o-Hydroxy-*trans*-cinnamoylglycine	—	274	324–325	—	18·4	10·3
o-Methoxy-*trans*-cinnamic acid	225	274	318–319	13·8	16·0	8·9
o-Glucosidurono-*trans*-cinnamic acid	225	274	310	12·8	17·6	8·4

were available, we have investigated qualitatively the action of the ascorbic acid–iron system and of Fenton's reagent on coumarin.

The experiments were carried out as follows. Coumarin (2·4 m-moles) was shaken at 37° in a 500 ml. flask with 100 ml. of phosphate buffer, pH 6·7. The solution was then saturated with oxygen gas and a mixture (10 ml.) containing $FeSO_4$, $7H_2O$ (0·52 m-mole), disodium salt of ethylenediaminetetra-acetic acid (2·5 m-moles) and ascorbic acid (5·6 m-moles) was added to the solution with shaking. The flask was stoppered and kept at 37° with constant shaking for 2 hr. Small samples were removed every 15 min. and chromatographed on paper. 7-Hydroxycoumarin was detected within 15 min. and was present throughout the 2 hr. period. The reaction mixture was extracted three times with 100 ml. portions of ether. The extract was evaporated and the small gummy residue was chromatographed in solvents *B* and *C* (Mead *et al.* 1958). The results are shown in Table 3. Fluorimetric determination of 7-hydroxycoumarin showed that 0·2 % of the coumarin had been oxidized to umbelliferone in 2 hr. The major products appeared to be 5- and 7-hydroxycoumarins, with traces of 6-hydroxycoumarin and *o*-coumaric acid. No 3-, 4- or 8-hydroxycoumarin or salicylic acid was found.

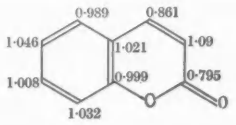
Similar experiments were carried out in which the coumarin was replaced by aniline, nitrobenzene, cyanobenzene and benzoic acid. The phenols formed were detected by colour reactions and paper chromatography and the results are shown in Table 4.

The experiments with Fenton's reagent and coumarin were carried out as described by Boyland & Sims (1953). The ether extract was chromatographed in solvents *A*, *B* and *C*. 3-, 5- and 7-Hydroxycoumarins were readily detected together with traces of 6- and 8-hydroxycoumarins and *o*-coumaric acid. 4-Hydroxycoumarin and salicylic acid were not found.

DISCUSSION

Metabolism of coumarin. From a quantitative point of view the recovery of coumarin as urinary metabolites (see Table 2) amounts to no more than 25 % of the dose (0·1 g./kg.), and at present we have no information regarding the rest of the material. Qualitatively, however, it has been shown that in the rabbit coumarin is hydroxylated in the 3-, 7- and 8-positions, and the impression is gathered from the amounts of the metabolites isolated that 3-hydroxycoumarin is the major hydroxylated product. No evidence was obtained of ring fission, and *o*-coumaric acid and its β-oxidation product, salicylic acid, were not found as metabolites. The above three hydroxycoumarins are also formed in the ferret, guinea pig, mouse and rat, and in the first three of these animals 5-hydroxycoumarin was found as well. Coumarin was not hydroxylated in the 4- or 6- positions in any of the species examined (see Table 3). The absence of 4-hydroxycoumarin supports the view that coumarin probably does not suffer ring fission to *o*-coumaric acid, since 4-hydroxycoumarin is a metabolite of this acid (see below).

The charge densities of the carbon atoms of coumarin have been given by Isaac (1955) (see below) and the positions of highest-charge density are in the descending order, 3-, 6- and 8-. Position 4 is one of low-charge density.



Electrophilic substitution of coumarin may therefore occur at positions 3-, 6- and 8-, whereas nucleophilic substitution occurs at 4. Now Fenton's reagent is regarded as a source of hydroxyl-free radicals and with this reagent some hydroxylation of coumarin occurs at all positions except 4 (see Table 3). *In vivo*, hydroxylation occurs at all positions except 4 and 6. The ascorbic acid–iron system, however, did not yield the 3-, 4- or 8-hydroxycoumarins. Neither of the systems *in vitro* produced the same results as those obtained *in vivo*, except that none of them produced 4-hydroxycoumarin. The ascorbic acid–iron system, however, did hydroxylate aniline, nitrobenzene and cyanobenzene in the same positions as found *in vivo* (see Table 4). The main difference between the hydroxylation of coumarin *in vivo* and by the ascorbic acid–iron system *in vitro* was the absence of 3-hydroxycoumarin formation by the system *in vitro*. This is interesting because when coumarin is incubated with rabbit-liver microsomes, suitably fortified, the formation of 7- but not of 3-hydroxycoumarin is readily detected (unpublished data). It is thus possible that the 3-hydroxylation of coumarin occurs by a different mechanism from 5-, 7- and 8-hydroxylation. This might be expected since the 3:4 double bond of coumarin possesses true double character, whereas those in the benzo ring are aromatic in character.

The precursors of the hydroxycoumarins *in vivo* could be 1:2-dihydro-1:2-diols. Four of these could be postulated, namely, the 3:4-, 5:6-, 6:7- and 7:8-dihydrodiols. On dehydration, these compounds would be expected to yield mainly 3-, 6- and 8-hydroxycoumarins with smaller amounts of 5- and

7-hydroxycoumarins, but practically no 4-hydroxycoumarin since the 4-position of coumarin is of low charge density. The formation of these diols in different amounts could in fact explain the formation *in vivo* of 3-, 5-, 7- and 8-hydroxycoumarins from coumarin.

Metabolism of o-*coumaric acid.* This acid (p*K* 4·6–4·7) is the *trans*-form of coumarinic acid (*o*-hydroxy-*cis*-cinnamic acid) which only exists as salts and normally occurs as the lactone, coumarin. We obtained no evidence that coumarin was converted *in vivo* into *o*-coumaric acid, but the latter on the other hand is converted into coumarin derivatives *in vivo*.

o-Coumaric acid, although it appears to be excreted unchanged to a considerable extent,

Table 2. *Conjugation of coumarin and* o-*coumaric acid*

Figures given are the averages for three animals (except where otherwise stated), the ranges being given in parentheses.

Compound fed	Dose (mg./kg.)	Percentage of dose excreted as			Umbelliferone excretion* (% of dose)		
		Glucuronide	Ethereal sulphate	Total conjugation	As glucuronide	As ethereal sulphate	Total
Coumarin	100	18 (17–18)	7 (6–7)	25	5 (3–6)[5]	6 (5–8)[6]	11
o-Coumaric acid	200	14 (9–17)	1 (0–2)†	14	—	—	—

* Umbelliferone excretion was determined in a separate set of experiments and the number of animals used is indicated by the superior figures outside the parentheses.

† These figures are within the experimental error of the method and are not significant.

Table 3. *Metabolites of coumarin in various species compared with the products of the action of ascorbic acid and of Fenton's reagent on coumarin*

Doses of coumarin: ferrets, 0·1 g./kg. orally with meat; guinea pigs, 0·14 g./kg. orally in gelatin capsules; mice, 0·4 g./kg. orally in arachis oil (this dose was fatal in 12–24 hr.); rabbits, 0·17 g./kg. orally in water; rats, 0·2 g./kg. orally in arachis oil or in gelatin capsules, and subcutaneously in arachis oil.

+, Indicates that the compound was readily detected by paper chromatography; −, the compound was not found; tr., the compound was detected in traces.

Metabolites sought	Metabolites found in the urine of					Products of the action of	
	Ferret	Guinea pig	Mouse	Rabbit	Rat	Ascorbic acid	Fenton's reagent
3-Hydroxycoumarin	+	+	+	+	+	−	+
4-Hydroxycoumarin	−	−	−	−	−	−	−
5-Hydroxycoumarin	tr.	+	+	−	−	+	+
6-Hydroxycoumarin	−	−	−	−	−	tr.	tr.
7-Hydroxycoumarin	+	+	+	+	+	+	+
8-Hydroxycoumarin	+	+	+	+	+	−	tr.
6:7-Dihydroxycoumarin	−	−	−	−	−	.	.
o-Coumaric acid	−	−	−	−	−	tr.	tr.
Melilotic acid	−	−	−	−	−	.	.
Salicylic acid	.	.	.	−	.	−	−

Table 4. *Hydroxylation of some monosubstituted benzenes by ascorbic acid–iron compared with that found* in vivo

+, Indicates that the phenol was formed; −, indicates that it was not; where negative results were obtained a check was made on the stability of the authentic phenol in the presence of the reagents. A blank space indicates that authentic phenol was not available for comparison.

Position of hydroxylation	Aniline		Nitrobenzene		Cyanobenzene		Benzoic acid.§
	Ascorbic acid–Fe	*in vivo**	Ascorbic acid–Fe	*in vivo*†	Ascorbic acid–Fe	*in vivo*‡	Ascorbic acid–Fe
ortho-	+	+	−	tr.	+	+	+
meta-	−	−	+	+	+	+	+
para-	+	+	+	+	+	+	+
2:3-	.	.	−	−	.	.	−
2:5-	.	.	+	+	.	.	+
3:4-	.	.	+	+	+	+	−

* Smith & Williams (1949); Parke & Williams (1956).

† Parke (1956).

‡ Smith & Williams (1950); Bray, Hybs & Thorpe (1951).

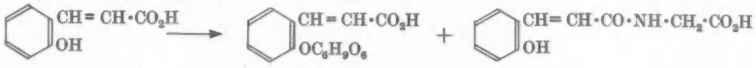
§ Benzoic acid is not hydroxylated *in vivo*.

undergoes two types of reactions *in vivo*, namely, direct conjugations at both the carboxyl and hydroxyl groups, and cyclization. Conjugation with glucuronic acid at the phenolic hydroxyl group occurs to a small extent and this was proved by the isolation of the non-reducing *o*-glucosiduronо-*trans*-cinnamic acid. There was no evidence that it formed an ethereal sulphate and in this respect it behaved like salicylic acid, of which it is a vinylogue. Conjugation at the carboxyl group was proved by the detection of *o*-hydroxy-*trans*-cinnamoylglycine in the urine. Whether or not it formed a reducing ester glucuronide (i.e. *o*-hydroxycinnamoyl glucuronide) was not proved. The urine was slightly reducing but this could have been due entirely to the reducing 4-glucosiduronocoumarin which was isolated from the urine. The conjugation reactions of *o*-coumaric acid appear to be those shown in scheme 1.

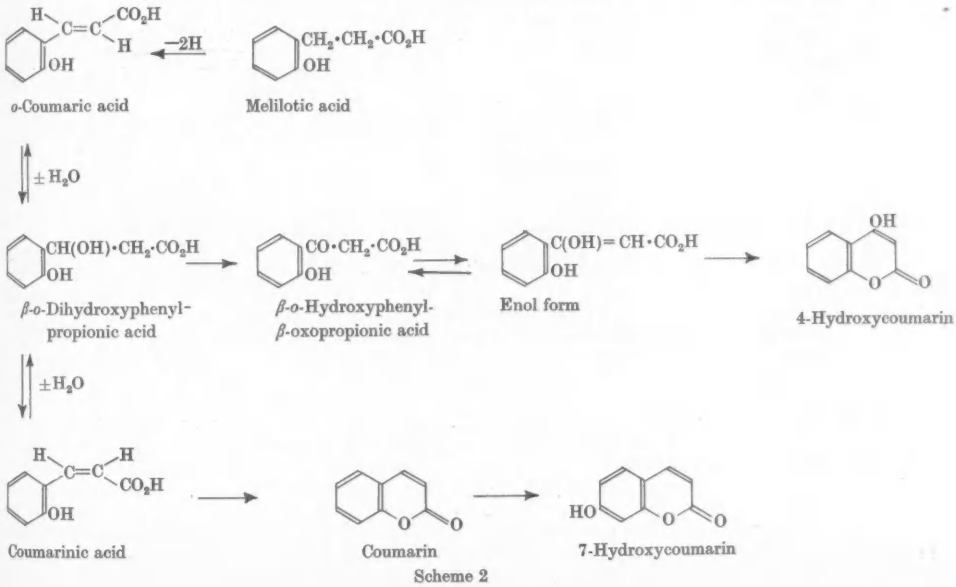
The more interesting aspect of *o*-coumaric acid metabolism is its conversion into the coumarin derivatives, 4- and 7-hydroxycoumarins, which involves a *trans–cis*-isomerization and a cyclization *in vivo*. The formation of the coumarin derivatives could be explained by assuming that *o*-coumaric acid undergoes the normal processes of β-oxidation (although it is not finally oxidized to salicylic acid) and that some of the intermediates of β-oxidation cyclize according to scheme 2.

In scheme 2 it is assumed that coumaric acid (and melilotic acid) undergoes β-oxidation as far as the keto acid stage, the enol form of the keto acid then cyclizing to 4-hydroxycoumarin. For the formation of 7-hydroxycoumarin, the intermediate formation of coumarin is tentatively postulated. Coumarin could be formed via coumarinic acid from β-*o*-dihydroxyphenylpropionic acid. If coumarin were formed, then 3- as well as 7-hydroxycoumarin could be expected, but only the 7-derivative was detected.

The occurrence in the urine of phenylketonurics of *ortho*-hydroxylated compounds derived from phenylalanine (see Boscott & Bickel, 1954) suggests that, in view of our findings with *o*-coumaric acid, it is not impossible that coumarin derivatives may be trace metabolites of phenylalanine and should be searched for.



Scheme 1



Scheme 2

SUMMARY

1. Coumarin when fed to rabbits is hydroxylated to 3-, 7- and 8-hydroxycoumarins, which are excreted in conjugation. This also occurs in the ferret, guinea pig, mouse and rat, the first three of which also excrete 5-hydroxycoumarin. However, not more than 25 % of the coumarin fed (dose 0·1 g./kg.) can be accounted for as conjugated glucuronic acid and ethereal sulphates excreted in the urine.

2. The ethereal sulphate and glucuronide of 3-hydroxycoumarin were isolated from the urine of rabbits fed on coumarin. 7-Hydroxycoumarin was also isolated after acid hydrolysis of the urine. 5- and 8-Hydroxycoumarins were not isolated but were detected in the hydrolysed urine by paper chromatography. The hydroxylation of coumarin has also been carried out with Fenton's reagent and an ascorbic acid–iron system. The results, which were not exactly the same as those found *in vivo*, have been discussed.

3. *o*-Coumaric acid appears to be excreted unchanged to a large extent by rabbits. It is, however, directly conjugated to a small extent since its ether glucuronide, *o*-glucosidurono-*trans*-cinnamic acid, was isolated and its glycine conjugate, *o*-hydroxy-*trans*-cinnamoylglycine, was detected by paper chromatography in the urine.

4. *o*-Coumaric acid is also cyclized *in vivo*, since small amounts of the glucuronide of 4-hydroxycoumarin were isolated from the urine and 7-hydroxycoumarin was detected chromatographically. Melilotic acid, the corresponding saturated acid (dihydrocoumaric acid), also yields 4- and 7-hydroxycoumarin in the rabbit.

5. The mechanism of the cyclization of *o*-coumaric acid has been discussed and it is suggested that the coumarin derivatives formed arise from β-oxidation products of *o*-coumaric acid.

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The Preparation and Properties of Lithium Hydroxypyruvate and Hydroxypyruvic Acid

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Interest in the possible biological importance of hydroxypyruvate has been revived by the recent demonstrations that it acts as an effective substrate for the L-lactic dehydrogenases of muscle (Meister, 1952) and yeast (Dickens & Williamson, 1956*a*), and for the specific D-glyceric dehydrogenase (hydroxypyruvate reductase) present in certain higher plants (Stafford, Magaldi & Vennesland, 1954), being thus reduced respectively to L- or D-glyceric acid. Hydroxypyruvate is also a substrate for transketolase (Horecker, Smyrniotis & Klenow, 1953; Haba, Leder & Racker, 1955) and for carboxylases of yeast (Dickens & Williamson, 1956*b*) and of muscle (Dickens & Williamson, unpublished work). A transaminase present in animal tissues is able to transfer the amino group of L-alanine to

hydroxypyruvate, yielding L-serine and pyruvate (Sallach, 1955, 1956). 3-Phosphohydroxypyruvate has also recently been synthesized (Ballou & Hesse, 1956) and its biological behaviour studied (Ichihara & Greenberg, 1957). Hitherto, pure and adequately characterized preparations of hydroxypyruvic acid or of its salts have not been available, and biological studies on hydroxypyruvate have been made directly on the solution obtained (Sprinson & Chargaff, 1946) by the action of two equivalents of sodium hydroxide on bromopyruvic acid (I). Such solutions contain, we find, besides the sodium bromide, sufficient unhydrolysed bromopyruvate to inhibit sensitive sulphydryl enzyme systems. Nevertheless, this work of Sprinson & Chargaff (which clearly indicates some of the serious uncertainties surrounding earlier preparations described as hydroxypyruvate) has been of great value in the subsequent biological studies.

The advantages of a pure crystalline salt of hydroxypyruvate, both for enzymic experiments and as an analytical standard, have now been achieved by the preparation of the lithium salt [Fig. 1 (II)], which proves to be the monohydrate, $CH_2(OH)\cdot C(OH)_2\cdot CO_2Li$. Evidence is provided in the Addendum by Bellamy & Williams that the above formula correctly represents the constitution of this salt, rather than $CH_2(OH)\cdot CO\cdot CO_2Li + H_2O$, since no free carbonyl group, or molecule of water of crystallization, is detected by infrared spectroscopy. It would therefore correspond in this respect with compounds such as chloral hydrate, $CH(OH)_2\cdot CCl_3$, as is discussed in the Addendum. These authors also find that a similar structure applies to the monohydrated lithium salt of pyruvic acid, $CH_3\cdot C(OH)_2\cdot CO_2Li$. In aqueous solutions containing ionized carboxyl groups these hydrated forms (diols) derived from the keto groups of the free acids will also be expected to be present.

Free hydroxypyruvic acid has not hitherto been obtained as an unequivocal preparation of the pure acid. The best crude preparations are probably those described by Sprinson & Chargaff (1946), by ether extraction made after acidification of concentrated aqueous solutions of the neutralized bromopyruvic acid. These oily products contained (by titration) 80–90 % of hydroxypyruvic acid. Fenton & Wilks (1912) prepared a 'basic lead salt' from the

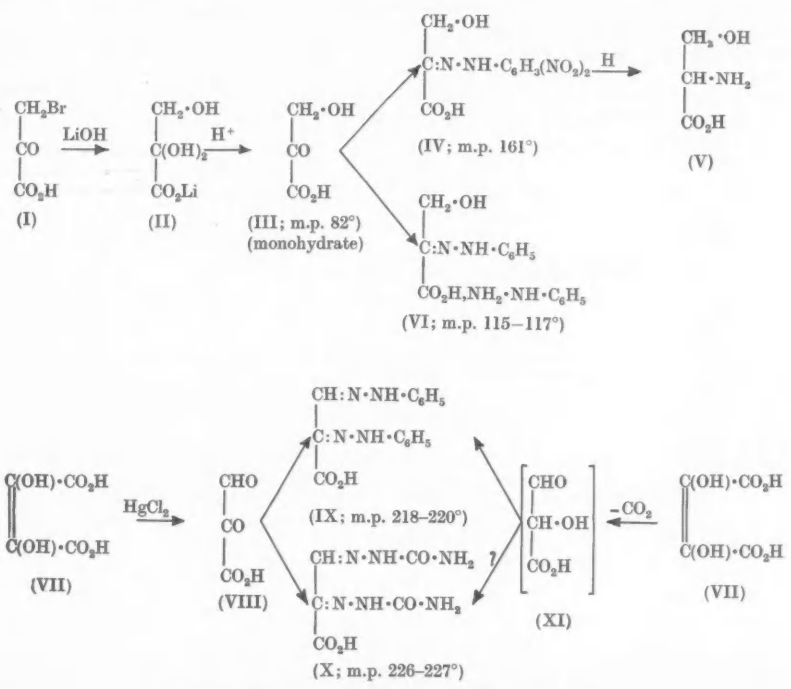

Fig. 1. All melting points are with decomposition.

crude oxidation mixture resulting from the action of hydrogen peroxide and ferrous salt upon glyceric acid. The composition assumed for this salt (and for the calcium salt also prepared by them) is based solely on the metal content of the hydrated salt, and is probably therefore unreliable. (The evidence based on their preparation of a semicarbazone compound is discussed below.) From this lead salt an acid was prepared by treatment with hydrogen sulphide and precipitation with petroleum from the ether solution. The compound was described as 'extremely hygroscopic' white crystals, $C_3H_4O_4$, 'either hydroxypyruvic acid, tartronic semialdehyde, or dihydroxyacrylic acid'. Fenton & Wilks (1912) favoured the view that this was hydroxypyruvic acid, but their evidence (based on the formation of glyceric acid by treatment with potassium cyanide of the lead salt derived from the crude glyceric acid oxidation mixture) is equivocal.

In order to clear up this confused situation, we have prepared, from the recrystallized lithium salt, analytically pure hydroxypyruvic acid monohydrate (III), which proves to be a white, non-hygroscopic solid of m.p. 82°. The properties of this acid ($C_3H_6O_5$) thus differ markedly from those of the product obtained by Fenton & Wilks. Infrared spectroscopy (see Addendum) indicates that the monohydrate of free hydroxypyruvic acid (unlike the salts) probably exists as the ketonic acid, two carbonyl groups—those of the keto and carboxylic groups—being detectable. The water molecule is, however, quite firmly attached and only slowly removed by heating in high vacuum over phosphoric oxide.

The identity of the lithium salt and free acid as being hydroxypyruvate was shown: (*a*) by the quantitative reduction of both compounds (to glyceric acid) by reduced diphosphopyridine nucleotide (DPNH) in the presence of both the lactic dehydrogenase of muscle and the specific hydroxypyruvate dehydrogenase of parsley leaves (Dickens & Williamson, 1958); (*b*) by quantitative decarboxylation with yeast carboxylase (cf. Dickens & Williamson, 1956*b*); (*c*) by means of a colour reaction with naphtharesorcinol (Dickens & Williamson, 1958); (*d*) by preparation of crystalline derivatives (see below).

The preparation of lithium hydroxypyruvate was mentioned in an earlier brief note (Dickens & Williamson, 1956*a*). The enzymic behaviour of these compounds will be presented in a forthcoming publication (cf. Dickens & Williamson, 1956*b*).

The infrared absorption is considered by Bellamy & Williams (see Addendum) to suggest that ionized solutions of both hydroxypyruvate and pyruvate will contain the hydrated derivatives rather than the free carbonyl-containing ketonic acids, whereas the latter may predominate in the un-ionized forms, such as will tend to be present at acidic reactions. This fact may be of importance in the enzymic behaviour of this group of substances. For muscle lactic dehydrogenase, Loewus, Ofner, Fischer, Westheimer & Vennesland (1953) have shown the transfer of hydrogen from one stereoisomer of DPNH to pyruvate to yield the resulting optically active lactate. Accepting a similar type of mechanism for hydroxypyruvate, it may be suggested that two different types of mechanism of reduction may occur, according to whether the hydrated pyruvate ion or the undissociated acid is the

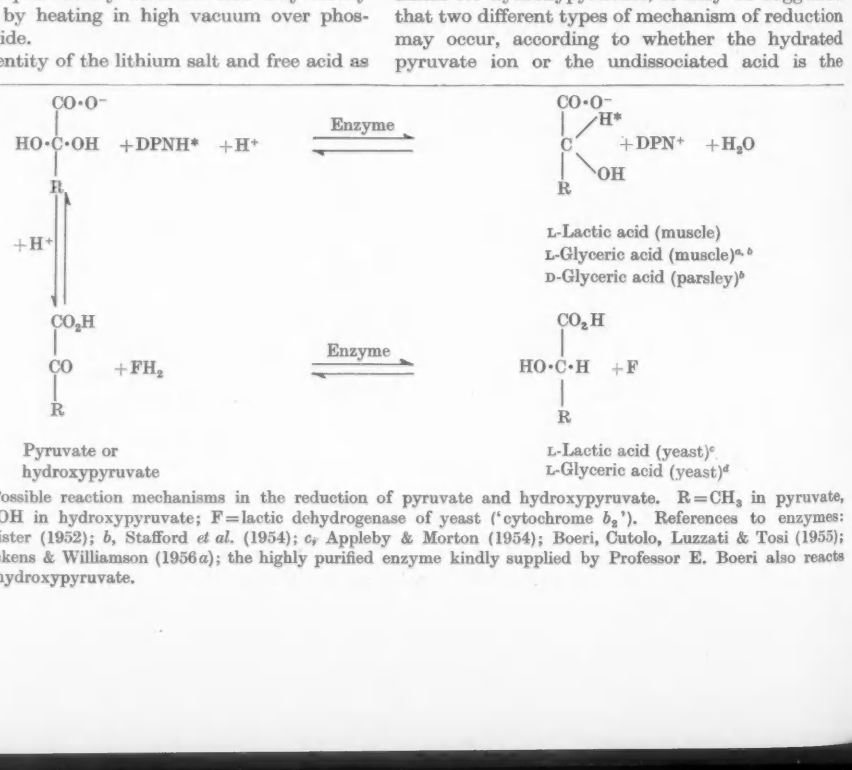

Fig. 2. Possible reaction mechanisms in the reduction of pyruvate and hydroxypyruvate. R = CH_3 in pyruvate, CH_2·OH in hydroxypyruvate; F = lactic dehydrogenase of yeast ('cytochrome b_2'). References to enzymes: *a*, Meister (1952); *b*, Stafford *et al.* (1954); *c*, Appleby & Morton (1954); Boeri, Cutolo, Luzzati & Tosi (1955); *d*, Dickens & Williamson (1956*a*); the highly purified enzyme kindly supplied by Professor E. Boeri also reacts with hydroxypyruvate.

reactant, the latter being presumably that favoured at acid pH values (see Fig. 2): the actual choice of pathways in Fig. 2 is somewhat arbitrary but is based roughly on the respective pH optima of the enzymes concerned.

EXPERIMENTAL

The melting points are uncorrected. All analyses are by Weiler and Strauss, Oxford. Pyruvic acid (British Drug Houses Ltd.) was twice fractionally distilled *in vacuo* and then it solidified readily in the refrigerator. Sodium [3-^{14}C]pyruvate and sodium [2-^{14}C]pyruvate were purchased from the Radiochemical Centre, Amersham, Bucks., with specific activity of approx. 2 mc/m-mole. Paper chromatography was on Whatman no. 1 paper with descending-liquid phase.

Preparation of 3-*bromopyruvic acid* (*I*). This is described for [3-^{14}C]bromopyruvic acid: the same method has been used for the [2-^{14}C]acid and (on four times the scale described below) for the unlabelled acid.

When the method of Sprinson & Chargaff (1946) was first used without modification, a long lag period was followed by a violently explosive bromination, with our materials. To avoid this danger, the addition of a single drop of conc. H_2SO_4 (cf. Ward, 1923) was found entirely satisfactory.

Twice-redistilled pyruvic acid (4·4 g., 50 m-moles) was added to the required quantity (e.g. 100 μc of specific activity 2·34 mc/m-mole) of sodium [3-^{14}C]pyruvate followed by 0·05 ml. of conc. H_2SO_4. With stirring and protection from moisture, 2·5 ml. of dry Br_2 was added slowly drop by drop while the flask was maintained in a bath at 50°. Under these conditions, the reaction began at once, with decolorization of the Br_2 as added and smooth evolution of HBr. The pale-yellow reaction mixture, after removal of HBr in a desiccator, set to a crystalline mass (97 % yield). After one recrystallization from hot dry $CHCl_3$ (1 ml./g.) colourless crystals, m.p. 58°, were obtained, having 70 % of the initial total radioactivity (mother liquors were discarded).

Preparation of lithium hydroxypyruvate monohydrate (*II*). Bromopyruvic acid (10 g., 60 m-moles) was dissolved in 100 ml. of water and N-LiOH was run in slowly with stirring until a pink colour to phenolphthalein persisted for 10 min. (approx. 90 % of that calc. for 2 moles of LiOH/mole of bromopyruvic acid). When this stage of neutralization was reached, 2 g. of activated charcoal was added, the filtrate adjusted to pH 5 by a few drops of acetic acid and concentrated under reduced pressure in N_2 to approx. 20 ml., when colourless crystals began to appear. These were collected after keeping the concentrate at 0° overnight, washed with 10 ml. of ice-cold water and dried over P_2O_5 *in vacuo* at room temperature: yield 3·7 g. (49 %). In spite of the loss on crystallization, attempts to recover pure material from the mother liquors were unsuccessful, no doubt because of the changes occurring on concentration of the aqueous solution (see below). Owing to the serious loss on recrystallization, *lithium* [^{14}C]*hydroxypyruvate* was generally used in this form without further purification (80 % of specific activity of bromopyruvic acid taken).

For recrystallization, the salt (II, 3·7 g.) was dissolved in 14 ml. of water at 65°, quickly centrifuged, and the decanted solution was kept at 0° overnight. Yield of colourless crystals after drying *in vacuo*, 1·65 g. [Found: C, 28·2; H, 3·9; Li, 5·36. $CH_2(OH)\cdot C(OH)_2\cdot CO_2Li$ requires C, 28·1; H, 3·9; Li, 5·4 %. Loss of wt. in high vacuum over P_2O_5: at 60°, no loss; at 110°, 0·0, 2·6 (two specimens). Calc. for 1 H_2O, 14·0 %; for 1 CO_2, 34·3 %].

The infrared absorption of this substance is described in the Addendum, and the colorimetric and enzymic assay of its content of hydroxypyruvic acid in the following paper (Dickens & Williamson, 1958). Its biological behaviour (cf. Dickens & Williamson, 1956*b*) will be fully reported later.

Preparation of hydroxypyruvic acid monohydrate (*III*). A solution of analytically pure lithium hydroxypyruvate monohydrate (500 mg.) in water (20 ml.) was shaken for 10 min. with Amberlite IR-120 (H^+ form; 5 g.). The filtrate and 10 ml. of water washings were combined and freeze-dried; the residual colourless syrup was kept over P_2O_5 in an evacuated desiccator. It was then dissolved in 15 ml. of ether at 0°, to the clear solution light petroleum (b.p. 60–80°) was slowly added to give a turbidity, with slight further additions over 2 days at 0°. The oil which first separated had then partially solidified and the supernatant liquid was decanted and discarded. To the solution of the precipitate in 15 ml. of acetone an equal volume of light petroleum (b.p. 60–80°) was added. After several days at 0°, the crystalline precipitate was collected and dried *in vacuo* over H_2SO_4 in the presence of paraffin-wax shavings. Yield, 160 mg. *Hydroxypyruvic acid monohydrate* had m.p. 81–82° (vigorous decomp.) [Found: C, 29·6; H, 4·90. $CH_2(OH)\cdot CO\cdot CO_2H,H_2O$ requires: C, 29·5; H, 4·92 %. (The anhydrous acid, $C_3H_4O_4$, would require C, 34·6; H, 3·84 %.) Loss of wt. at 0·05 mm. Hg over P_2O_5 at 56°: 2 hr. 8·0; 4 hr. 9·4; 6 hr. 11·1; 8 hr. 11·2. Calc. for 1 H_2O, 14·75 %]. On titration in N_2 with $Ba(OH)_2$ and phenolphthalein, an equivalent wt. of 111 was obtained (calc. 122); the low value is doubtless due to alkaline decomposition (see below). After drying for 8 hr. as described above, the m.p. (85°, decomp.) showed little change, although decomposition is shown by the loss of weight which had occurred.

The infrared absorption spectrum of this compound is reported in the Addendum.

Preparation of the 2:4-*dinitrophenylhydrazone of hydroxypyruvic acid* (*IV*) *from the lithium salt.* Lithium hydroxypyruvate monohydrate (II; 50 mg.) was dissolved in 2 ml. of water and 20 ml. of a cool saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl was added. The yellow crystals which separated almost immediately were collected after keeping at 0° overnight, washed with water and dried *in vacuo* over P_2O_5. Yield, 105 mg. (92 %), m.p. crude 135–138° (decomp.); after recrystallization from ethyl acetate–light petroleum (3:1) the m.p. was 160–161° (decomp.) (Sprinson & Chargaff, 1946, give m.p. 162°). After electrolytic reduction (for which we are indebted to Dr I. Smith) and paper chromatography in butanol–acetic acid–water mixture (4:1:5, by vol.) the presence of serine (V) (cf. Sprinson & Chargaff, 1946) was identified by comparison with a marker.

Attempted preparation of a phenylosazone from lithium hydroxypyruvate. The lithium salt (1 m-mole) was dissolved in N-HCl (1 equiv.) and phenylhydrazine (3 m-moles) was added (pH about 5). The solution was kept at 80°; after 5 min. an orange precipitate formed and this was collected after 20 min. at 80°, washed with water and dried (143 mg., 61 %, m.p. 115–117°, decomp.). After precipitation from $CHCl_3$ by light petroleum (b.p. 60–80°), the m.p. was

unchanged (Found: C, 60·2; H, 5·7; N, 18·6. The bisphenylhydrazone $C_{15}H_{14}O_2N_4$ would require C, 63·8; H, 5·0; N, 19·8. The phenylhydrazide of hydroxypyruvic acid monophenylhydrazone (VI), $C_{15}H_{16}O_3N_4$, requires C, 59·7; H, 5·95; N, 18·5%).

Other variations of this preparation failed to give the crystalline osazone (IX), m.p. 207°, reported by Fenton & Jones (1900): the m.p. was later raised (Fenton & Ryffel, 1902) to 222–224°. It is noteworthy that Sprinson & Chargaff (1946), from their solutions of alkali hydroxypyruvate, were also unable to obtain this compound on the addition of phenylhydrazine acetate, but obtained only 'brown amorphous products' or at higher temperatures 'deeply coloured products' quite unlike the Fenton & Jones osazone.

Attempted preparation of the semicarbazone from lithium hydroxypyruvate. The aqueous solution of recrystallized lithium hydroxypyruvate was treated at room temperature with excess of semicarbazide hydrochloride, with or without the addition of sodium acetate. After several days no precipitation occurred. After evaporation of the solution to small volume only semicarbazide hydrochloride could be recovered. This behaviour also contrasts strongly with that described by Fenton & Wilks (1912) for their oxidation product from glyceric acid, which gave an insoluble substance, m.p. 221°, which they believed (from the analysis only) to be the semicarbazide salt of the semicarbazone of hydroxypyruvic acid,

$$CH_2(OH)\cdot C(:N\cdot NH\cdot CO\cdot NH_2)\cdot CO_2H,NH_2\cdot NH\cdot CO\cdot NH_2.$$

Although a similar compound did not arise from our preparation of lithium hydroxypyruvate, no difficulty was experienced in preparing a compound of this m.p. from dihydroxyfumaric acid and semicarbazide hydrochloride as described by Fenton & Wilks (1912). For this purpose, recrystallized dihydroxyfumaric acid dihydrate (VII; 5 m-moles) was dissolved with gentle warming in a solution of semicarbazide hydrochloride (20 m-moles in 60 ml. of water). After 16 hr. at room temperature the copious white precipitate (m.p. crude 210°, decomp.) was recrystallized from hot acetic acid, and dried for 30 min. at 100°, at atmospheric pressure; m.p. 227° (decomp.) (Found: C, 26·27; H, 4·43; N, 35·56. Calc. for $C_5H_8O_4N_6,H_2O$ (X): C, 25·7; H, 4·30; N, 35·9%). The compound was dried at 80° in high vacuum over P_2O_5 for 5 hr. (Found: C, 26·8; H, 4·17. Calc. for $C_5H_8O_4N_6$, 0·5 H_2O: C, 26·6; H, 4·0%). Fenton & Wilks (1912) state that the compound obtained from dihydroxyfumaric (dihydroxymaleic) acid by the above treatment is identical with that obtained from their oxidation product derived from glyceric acid: this latter substance had m.p. 221° after recrystallization from acetic acid and its analysis gave C, 25·4; H, 4·68; N, 35·5. Calc. for $C_5H_{12}O_5N_6$: C, 25·4; H, 5·07; N, 35·6%. Fenton & Wilks attributed this to the semicarbazide salt of the semicarbazone. On the other hand, our analysis (above) agrees better with the monohydrated formula X, ($C_5H_{10}O_5N_6$) which loses 0·5 H_2O on further drying. This would suggest that the Fenton & Wilks compound may have been the disemicarbazone of mesoxalic semialdehyde (VIII), containing a molecule of water, which, as our analyses show, is very difficult to remove from this compound. In order to obtain further evidence on this point: (*a*) the compound, m.p. 227° decomp., was refluxed with water containing a little benzoic acid to which excess of benzaldehyde was added, with a view to removing the molecule of semicarbazide supposedly present as the salt of the carboxyl group. No decomposition occurred and the unchanged substance (m.p. 215–217°, decomp.; after one recrystallization, m.p. 225°, decomp.) was recovered. (*b*) The disemicarbazone of mesoxalic acid semialdehyde was prepared as described below, m.p. 225–226° (decomp.), and after drying at 80° *in vacuo* over P_2O_5 gave analysis for C, H and N corresponding closely with the formula $C_5H_8O_4N_6$,0·5 H_2O, identical with that obtained on the substance, m.p. 227° (decomp.), prepared from dihydroxyfumaric acid.

It is therefore considered probable that the Fenton & Wilks compound, m.p. 221°, was in fact the hydrated disemicarbazone of mesoxalic acid semialdehyde and not that of the semicarbazide salt of hydroxypyruvic acid semicarbazone, as these authors believed it to be. The precipitation of the phenylosazone (m.p. 209°) of tartronic acid semialdehyde (XI) occurs immediately when phenylhydrazine reacts at room temperature with XI (Fischer, Baer & Nidecker, 1937). If a similar reactivity to semicarbazide is postulated, this substance (XI) could well be the intermediate in reactions VII → IX and VII → X as is suggested in Fig. 1.

These considerations shed doubt on the purity of the product obtained by oxidation of glyceric acid by Fenton & Wilks (1912), and used by them in preparing the osazone, which neither we nor Sprinson & Chargaff (1946) could obtain from solutions of hydroxypyruvate. Nor does our purified lithium hydroxypyruvate give the insoluble semicarbazide derivative of m.p. 221° obtained by Fenton & Wilks from their material.

Preparation of mesoxalic acid semialdehyde and its derivatives. Dihydroxyfumaric acid dihydrate (VII) was oxidized with $HgCl_2$ at 60° as described by Fenton (1905). After removal of HgCl by filtration and of a trace of excess of Hg^{2+} ions by H_2S, the filtrate and washings were concentrated in an evacuated desiccator over P_2O_5 and NaOH to give a solution of mesoxalic acid semialdehyde (VIII) containing approx. 0·4 m-mole/ml.

Bisphenylhydrazone of mesoxalic acid semialdehyde (*IX*). This was prepared from a portion of the above solution at room temperature by addition of phenylhydrazine (4 mol. prop.; pH of mixture approx. 5). Immediate precipitation occurred of the yellow osazone in good yield, m.p. crude 147° (decomp.), after recrystallization from hot $CHCl_3$ and a little acetic acid, m.p. 218–220° (decomp.) (Found: N, 20·2. Calc. for $C_{15}H_{14}O_2N_4$: 19·9%). The m.p.'s recorded from the literature by Fenton & Ryffel (1902) for this much studied osazone ('the osazone of hydroxypyruvic acid') vary from 201–203° to 222–224°. The same compound is obtained by heating dihydroxymaleic acid with phenylhydrazine (Fenton & Ryffel, 1902): by this method we found m.p. 218–219° (decomp.). The product obtained from mesoxalic acid semialdehyde was converted into the pyrazolone (Dakin, 1917) by heating with ethanolic HCl, m.p. 146° (Dakin gives m.p. 150°) (Found: N, 21·0. Calc. for $C_{15}H_{12}ON_4$: N, 21·2%).

Disemicarbazone of mesoxalic acid semialdehyde (*X*). This was prepared from a further portion of the same solution of mesoxalic acid semialdehyde. On addition of semicarbazide hydrochloride precipitation occurred immediately (yield, 65% of that calc. on original wt. of dihydroxyfumaric acid taken); the product, after recrystallization from hot acetic acid, had m.p. 225–226° (decomp.) [Found:

(dried at 80° *in vacuo* over P_2O_5) C; 26·5; H, 4·37; N, 37·2. Calc. for $C_3H_3O_4N_3$,0·5 H_2O: C, 26·6; H, 4·0; N, 37·3%. Loss of wt. after 2 hr. at 110° at 0·01 mm. Hg over P_2O_5: 10·8. 1 H_2O requires 7·7%]. After the 2 hr. drying, m.p. was 220° (decomp.); evidently some decomposition had occurred.

Preparation of lithium pyruvate monohydrate. For spectrographic purposes (see Addendum) a sample of lithium salt was prepared from twice redistilled pyruvic acid by a convenient modification of the method of Wendel (1932). The well-stirred aqueous solution of pyruvic acid (0·1 mole in 10 ml.) was titrated with 3·33 N-LiOH until just blue to bromothymol blue. Crystals rapidly began to separate, and after 16 hr. at 0° they were collected, washed with a little ice-cold water, and dried *in vacuo* over $CaCl_2$. Yield, 4·2 g. Recrystallization from 15 ml. of water at 90° gave 2·7 g. (Found: C, 32·0; H, 4·4; Li, 5·97. Calc. for $CH_3{\cdot}CO{\cdot}CO_2Li,H_2O$: C, 32·15; H, 4·46; Li, 6·25%. No loss of wt. at 110° in high vacuum over P_2O_5).

Paper chromatography of hydroxypyruvate. Milhaud, Benson & Calvin (1956) have reported two-dimensional paper chromatograms obtained with unpurified [2-^{14}C]-hydroxypyruvate obtained from the bromopyruvate. In phenol–water (4:1, v/v) two distinct spots were obtained, R_F 0·15 and 0·31.

Recrystallized lithium hydroxypyruvate was chromatographed in the above liquid and also in butanol–acetic acid–water (4:1:5, by vol.). The aniline–phosphoric acid mixture of Bryson & Mitchell (1951) used for detection was sprayed on the dried papers, which were then heated for 5 min. at 100°. Single compact purplish-brown spots resulted, obtainable with amounts down to 10 μg. of hydroxypyruvate, and having R_F in phenol–water 0·08, in butanol–acetic acid–water, 0·22.

Further characterization is possible by means of the dinitrophenylhydrazone, m.p. 160–161°, described above. A solution in 0·01 M-phosphate, pH 7·4, was chromatographed with butanol–ethanol–water (Cavallini & Frontali, 1954) as solvent. The hydrazone gave a single yellow spot, R_F 0·32.

Effect of heat on solutions of hydroxypyruvate

Fenton & Wilks (1912) stated briefly that solutions of hydroxypyruvic acid are destroyed by heating on a water bath, a fact which 'sharply distinguishes it from Will's acid' (cf. Will, 1891).

Solutions of lithium hydroxypyruvate were heated at various temperatures and pH values. When the pure aqueous solution of the salt was heated in a boiling-water bath, the reaction rapidly became strongly alkaline owing to lithium carbonate formation, and after 30 min. at 100° only a trace of the original hydroxypyruvate could be detected colorimetrically or enzymically. Paper chromatography of the heated solution showed the presence of a new reducing spot, which was identified as erythrulose (XII) by paper chromatography of the heated deionized solution in phenol–water and in butanol–acetic acid–water (R_F 0·71 and 0·35 respectively; L-erythrulose 0·71 and 0·35; D-erythrose 0·64 and 0·36). For further characterization the deionized heated solution was evaporated *in vacuo* to a syrup (100 mg. from 640 mg. of lithium salt taken) which was dissolved in 3 ml. of ethanol, filtered and warmed for 5 min. in a boiling-water bath with *o*-nitrophenylhydrazine (110 mg.). The filtered solution was diluted with water, the hot solution was treated with charcoal, and, on cooling, orange-yellow crystals separated, which were washed with ether, dried and recrystallized from hot water. Yield, 17 mg., m.p. 123–125° (Found: N, 16·1. Calc. for erythrulose *o*-nitrophenylhydrazone 16·5%). The substance appears to be this derivative of DL-erythrulose; the corresponding derivative of L-erythrulose has m.p. 152° (Müller, Montigel & Reichstein, 1937). A portion was decomposed by refluxing with water containing benzoic acid and benzaldehyde. After removal of these substances the solution of the free sugar was chromatographed and run together with authentic L-erythrulose. However, on this particular chromatogram, erythrose could not be clearly distinguished from erythrulose, so that some erythrose may possibly have been present. The amount of material was insufficient for a fuller examination. Table 1 (Tube A) shows the estimations of rate of formation of products in the above experiment.

In acid solutions (0·5 M-acetate buffer, pH 5·5; 0·1 M-acetic acid solution) at 100° decarboxylation also occurs. In the pH 5·5 buffer the solution again, but more slowly, became alkaline, and the rate of breakdown was similar to that of the heated lithium salt solution. In acetic acid it was still slower. Analysis of the heated mixtures (Table 1) was by (*a*) the colorimetric method (Dickens & Williamson, 1958); (*b*) by measurement of the decarboxylation with a preparation of yeast carboxylase (Dickens & Williamson, 1956*b*); (*c*) by the hydroxypyruvate reductase of Stafford *et al.* (1954). Method (*a*) estimates both hydroxypyruvate and glycolaldehyde, methods (*b*) and (*c*) only hydroxypyruvate. It will be seen from Table 1 that whilst the three methods give closely similar values for the heated aqueous solution of lithium hydroxypyruvate (pH 6·5 → 9), there is a serious discrepancy in the values as obtained colorimetrically after heating at pH 5·5 → 8·1, and still more so in the acetic acid solution, when these are compared with the enzymic assays. This difference is to be attributed to the accumulation of glycolaldehyde (XIII), which does not occur in the series at pH 6·5 → 9·0. That this is the source of the differences found is shown by the figures in parentheses under 'colorimetric' in Table 1. These values are corrected for the presence of glycolaldehyde found after absorption of hydroxypyruvic acid on to Bio-Deminrolit G resin according to the method of Dickens & Williamson (1958). The corrected values for hydroxypyruvate agree quite closely with those determined enzymically.

From these experiments it is clear that, of the reactions

$$\underset{\text{(III)}}{CH_2(OH){\cdot}CO{\cdot}CO_2H} \rightarrow \underset{\text{(XIII)}}{CH_2(OH){\cdot}CHO} + CO_2 \quad \text{and}$$

$$2\underset{\text{(II)}}{CH_2(OH){\cdot}C(OH)_2{\cdot}CO_2Li} \rightarrow \underset{\text{(XII)}}{CH_2(OH){\cdot}CO{\cdot}CH(OH){\cdot}CH_2{\cdot}OH} + 2LiHCO_3,$$

the former decarboxylation proceeds slowly at an acid pH whilst the latter condensation predominates at neutral and alkaline reactions and is then quite rapid. The resemblance of this chemical decarboxylative ketol condensation to the similar enzymic formation of erythrulose from hydroxypyruvate (Dickens & Williamson, 1956*b*) is quite striking; the main difference is that the enzymic reaction, involving carboxylase and transketolase, yields the pure L-erythrulose, whereas this reaction, of course, gives the DL-form.

Table 1. *Effect of heating aqueous solutions of hydroxypyruvate*

Each of three stoppered test tubes (*A*, *B* and *C*) contained 64 mg. (500 μmoles) of lithium hydroxypyruvate: *A*, in 5 ml. of water; *B*, in 5 ml. of 0·5 M-acetate, pH 5·5; *C*, in 5 ml. of 0·1 N-acetic acid. The tubes were heated in a boiling-water bath and samples removed for analysis as detailed in the text; colorimetrically and by hydroxypyruvate reductase (see Dickens & Williamson, 1958); by yeast carboxylase and measurement of CO_2 manometrically (see Dickens & Williamson, 1956*b*).

Results are expressed in μmoles of hydroxypyruvate/ml. of solution. The values in parenthesis are the amounts of hydroxypyruvate found colorimetrically after deduction of the glycolaldehyde present (non-absorbed on Bio-Deminrolit G, see Dickens & Williamson, 1958).

Tube and pH	Time at 100° (min.)	Hydroxypyruvate found (μmoles) Colorimetric method	Carboxylase method	Reductase method
A (pH 6·5→9)	0	102	93	95
	5	100	90	95
	10	76	74	72
	20	17·5	16·5	16·5
	30	1	3·5	2·5
B (pH 5·5→8·1)	0	98	95	95
	10	72	48	45
	20	41	16	16·5
	40	17	3	2·5
	60	9	0	0
C (0·1 N-acetic acid)	0	98 (95)	93	94
	10	96 (93)	90	86
	20	93 (84)	83	84
	40	86 (71)	73	72
	60	82 (62)	60	60

Action of cold dilute alkali on hydroxypyruvate

Sprinson & Chargaff (1946) reported that solutions of NaOH (0·1–1·5 N) acting on hydroxypyruvate yielded an unsaturated compound which, as shown by iodine titration, could be accounted for by a 35–64% formation of the enediol:

$$CH_2(OH)\cdot CO\cdot CO_2^- \xrightarrow{OH^-} C(OH)=CH(OH)\cdot CO_2^-.$$

We have attempted to prepare this compound (dihydroxyacrylic acid) but without success. When a lithium hydroxypyruvate solution in N-LiOH was kept under N_2 for 1 hr. at 20°, deionized with Amberlite 1R-120(H^+) and freeze-dried, a solid semicrystalline residue was obtained. This was washed with cold acetone and dried *in vacuo* (yield 30% of wt. of lithium salt taken). It consisted of a non-hygroscopic white solid, m.p. (unsharp) about 85° (decomp.) (Found: C, 30·7; H, 4·2. $C_3H_3O_3$ requires C, 30·6; H, 4·1%). Titration gave two different values according to the conditions of titration, corresponding to equiv. wts. of 226 and 522 respectively; the lower value was obtained with $Ba(OH)_2$ in N_2 and the higher with alkali hydroxide in air. The substance is thus not dihydroxyacrylic acid, and in agreement with this did not decolorize iodine solution. The analysis would correspond with that of an addition product of hydroxypyruvic acid and glyoxylic acid (mol.wt. 196), or a higher polymer of similar composition. It was not further investigated.

SUMMARY

1. The preparation of crystalline lithium hydroxypyruvate is described, together with the application of the method for preparing lithium [^{14}C]hydroxypyruvate.

2. The lithium salt has the analysis of the monohydrate, $CH_2(OH)\cdot C(OH)_2\cdot CO_2Li$, in which the molecule of water is very firmly bound.

3. Hydroxypyruvic acid, m.p. 81–82° (decomp.), has been prepared from the lithium salt and is also a monohydrate, $CH_2(OH)\cdot CO\cdot CO_2H,H_2O$; it thus differs from the compound described as hydroxypyruvic acid by Fenton & Wilks (1912).

4. Lithium hydroxypyruvate yields the characteristic dinitrophenylhydrazone, giving serine on reduction. It gives no osazone under the usual conditions, but a crystalline derivative, which may be the phenylhydrazide of the phenylhydrazone, is obtainable. The reasons for the apparently different behaviour of the Fenton & Wilks compound are discussed. It is suggested that the semicarbazide compound obtained by these authors may have been the hydrated disemicarbazone of mesoxalic acid semialdehyde and not, as they assumed, the semicarbazide salt of hydroxypyruvic acid monosemicarbazone.

5. The nature of lithium hydroxypyruvate and hydroxypyruvic acid have been investigated colorimetrically, enzymically, chromatographically and (see Addendum) by infrared spectrophotometry.

6. When aqueous solutions of hydroxypyruvate are heated, carbonate is liberated and a reducing substance which is believed to be DL-erythrulose has been demonstrated as a major product. The rate of decomposition of hydroxypyruvate at 100° and various pH ranges has been studied. In acidic

solutions the thermal decomposition is slower and the main product then reacts as glycolaldehyde.

7. The action of N-alkali on hydroxypyruvate yields an unidentified polymerized decomposition product $(C_3H_4O_3)_n$ which is not the unknown dihydroxyacrylic acid.

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ADDENDUM

The Infrared Spectra of Hydroxypyruvic Acid and Related Compounds

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In connexion with the work described in the previous paper we have examined the infrared spectra of pyruvic acid, hydroxypyruvic acid monohydrate and their hydrated lithium salts. The spectra are illustrated in Fig. 1 and are also discussed below.

Pyruvic acid has been previously studied by Randall, Fuson, Fowler & Dangl (1949) and by Thomas (1955), and the spectrum has been reported to be abnormal in that only a single carbonyl frequency is shown despite the presence of the two carbonyl functions. Under reasonably high resolution we find that two peaks are in fact present in the liquid, at 1773 cm.$^{-1}$ and 1716 cm.$^{-1}$, although the intensity relation is such that the higher-frequency band appears as a well-defined shoulder on the side of the 1716 cm.$^{-1}$ band. In chloroform solution the two bands are well resolved and occur at 1788 cm.$^{-1}$ and 1736 cm.$^{-1}$. The very high frequency of the first of these absorptions is not easy to understand as α-diketones do not normally show appreciable interaction effects (Bellamy, 1954). The most likely explanation would seem to be that the possibility of intramolecular hydrogen bonding between the CO_2H and α-keto groups allows the liquid acid to exist as the monomer in the liquid rather than as the more usual dimer, which would absorb at lower frequencies (Bellamy, 1954; Flett, 1951). This is supported by the behaviour in dioxan solution, in which dimeric structures are usually broken (Flett, 1951). In this case no new high-frequency band is found and the absorption (1769 cm.$^{-1}$) is essentially unchanged. The lower-frequency (keto) band moves to 1733 cm.$^{-1}$ and a third band of intermediate

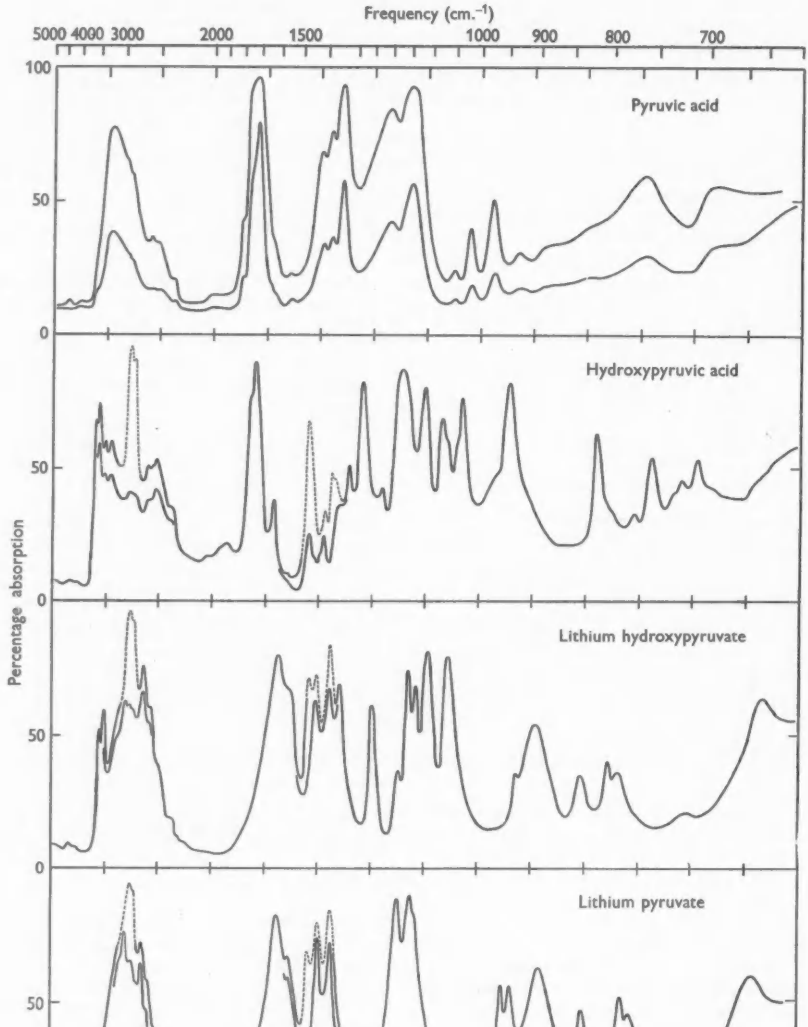

Fig. 1. Infrared absorption of pyruvic and hydroxypyruvic acids and of their lithium salts. The spectrum of pyruvic acid is that of two capillary films of different thickness. The spectra for the remaining three substances are for mulls in Nujol, the region of absorption due to the Nujol being shown by the dotted lines. The solid line beneath each dotted section is the spectrum of the substance as a mull in Florube (a fully fluorinated hydrocarbon having no absorption in the dotted regions).

frequency appears at 1753 cm.$^{-1}$. This is typical of the frequency which would be expected from a monomeric acid bonded intermolecularly to the solvent rather than intramolecularly to itself. Further, the intensity changes in this band and that at 1733 cm.$^{-1}$ which follow alterations in concentration or in solvent indicate clearly that the intensity of the new band increases when that of the 1770 cm.$^{-1}$ band diminishes, and vice versa, whereas the intensity of the 1733 cm.$^{-1}$ absorption remains essentially unchanged. Finally, the principal OH stretching frequency of the liquid acid is at 3220 cm.$^{-1}$ which is much higher than is usual in dimers (Bratoz, Hadzi & Sheppard, 1956). Pyruvic acid can therefore be regarded as a normal α-keto acid and the unusual features of its infrared spectrum can be attributed to its existence in the liquid state as an intramolecularly bonded monomer.

The spectrum of the hydrated lithium salt of pyruvic acid is in marked contrast with that of the parent acid. No carbonyl absorptions are shown but the characteristic CO_2^- stretching bands of the ionized acid are well marked at 1608 cm.$^{-1}$ and 1435 cm.$^{-1}$. In addition to the changes to be expected from the removal of the un-ionized carboxyl group, marked alterations occur in other regions of the spectrum. In particular, strong bands appear from 1100 to 1000 cm.$^{-1}$, a region in which bands arising from OH deformation modes commonly occur. In the high-frequency region, bands due to OH stretching modes are found at 3100 cm.$^{-1}$ (shoulder) and at 2700 cm.$^{-1}$ with a complex pattern of bands in the range 2700–2000 cm.$^{-1}$. These are characteristic of very strongly bonded hydroxyl groups and are not commonly found in ionized acids. Also they occur at very much lower frequencies than is normally associated with water of crystallization (see below). The absence of any ketonic function and the presence of OH groups is consistent with the structure $CH_3 \cdot C(OH)_2 \cdot CO_2^- Li^+$ and excludes the alternative formulation $CH_2 \cdot CO \cdot CO_2^- Li^+, H_2O$.

The spectra of the hydrates of hydroxypyruvic acid and its lithium salt are essentially parallel to the above. In the solid acid two distinct carbonyl absorptions can be clearly identified at 1757 cm.$^{-1}$ and 1730 cm.$^{-1}$, which probably originate in the carboxylic acid group (monomer) and ketonic carbonyl respectively. In the high-frequency region four bands are found above 3000 cm.$^{-1}$. Of these the high-frequency bands at 3509 cm.$^{-1}$ and 3448 cm.$^{-1}$ probably originate in the CH_2OH group and possibly also in the monomeric CO_2H, whereas the lower-frequency bands at 3333 cm.$^{-1}$ and 3205 cm.$^{-1}$ probably arise from the asymmetric and symmetric stretching modes of the associated water molecule. The positions of bands from hydrated water in this region vary to some extent with the strengths of the hydrogen bonds by which the water is attached, but two frequencies in this region are shown, for example, by many hydrated inorganic salts and similar materials (Lecomte, 1953). Finally, a band is present at 1623 cm.$^{-1}$ which is absent from the spectrum of pyruvic acid. This is the OH deformation frequency of bonded water. The spectrum of this acid therefore indicates that it retains its ketonic character in the solid state.

In the hydrated lithium salt of hydroxypyruvic acid there are again drastic modifications of the spectrum. In the high-frequency region the absorptions at 3120 cm.$^{-1}$ and 2703 cm.$^{-1}$ and the series of minor peaks between 2700 and 2000 cm.$^{-1}$ reappear as in lithium pyruvate monohydrate. These are almost certainly OH stretching modes and combination bands associated with the $-C(OH)_2-$ group. In addition, two bands appear at 3436 cm.$^{-1}$ and 3311 cm.$^{-1}$. These are absent from the spectrum of lithium pyruvate, and probably both of them are associated with the $CH_2 \cdot OH$ end-group. The origin of the doubling is not clear but crystal-splitting effects may be involved. As before, no ketonic carbonyl absorptions can be detected and the strong CO_2^- bands are shown at 1605 cm.$^{-1}$ and 1429 cm.$^{-1}$. This spectrum is therefore consistent only with the structure

$$CH_2(OH) \cdot C(OH)_2 \cdot CO_2^- Li^+.$$

It would therefore seem that a clear-cut distinction exists between the nature of any bound water in the free acids and their lithium salts. In the former any hydration which occurs does so as water of crystallization, whereas in the latter addition occurs across the carbonyl link to form the $-C(OH)_2-$ group. This is not unexpected in view of the considerable differences in the inductive effects of the ionized and un-ionized carboxyl group. In compounds such as chloroacetone and fluoropyruvic acid, the electronegativity of the CH_2Cl and CH_2F groups is insufficient to lead to hydration across the carbonyl link, and in the latter the ketonic absorption is clearly seen in the infrared spectrum as it is shifted to higher frequencies (L. C. Thomas, personal communication). On the other hand, in compounds such as chloral hydrate and α-thenoyltrifluoroacetone hydrate which we have also studied, the electronegativities of the CCl_3 and CF_3 groups are sufficient to lead to addition across the C=O bond and the total disappearance of the appropriate carbonyl absorption from the infrared spectrum.

It should be noted, however, that the distinction between the free acids and their salts which we have observed is primarily of importance in the solid state. In aqueous solutions some degree of ionization of the carboxyl group will occur which would at once be followed by hydration of the ketonic

grouping, to the diol. Hydroxypyruvic acid in aqueous solution might therefore be expected to have a potential ability to react in either of the two forms

$CH_2(OH)\cdot CO\cdot CO_2H$ and $CH_2(OH)\cdot C(OH)_2\cdot CO_2H$.

Experimental. Spectra were measured with a Grubb Parsons S. 3A double-beam spectrometer with a sodium chloride prism. Samples were examined as capillary films or as thin mulls in paraffin and in fluorinated paraffin oil.

SUMMARY

1. The infrared-absorption spectra of pyruvic acid, hydroxypyruvic acid monohydrate and their monohydrated lithium salts are reported.

2. Pyruvic acid can be regarded as a normal α-keto acid except for features in its spectrum suggesting intramolecular bonding.

3. Lithium pyruvate monohydrate has a quite different spectrum, no ketonic carbonyl absorption being detectable but CO_2^- stretching bands are well marked, as well as bands characteristic of strongly bonded hydroxyl groups. The spectrum is consistent with the formulation $CH_3\cdot C(OH)_2\cdot CO_2^-Li^+$ and not with $CH_3\cdot CO\cdot CO_2^-Li^+,H_2O$.

4. The spectra of the hydrates of hydroxypyruvic acid and of its lithium salt are parallel with the above, and indicate that whereas the free acid retains its ketonic character in the solid state and the hydration is present as water of crystallization, the lithium salt shows no ketonic carbonyl absorption and its spectrum is consistent only with the structure $CH_2(OH)\cdot C(OH)_2\cdot CO_2^-Li^+$.

We are extremely grateful to Professor F. Dickens for making these interesting materials available to us for study.

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The Determination of Hydroxypyruvate and Glycolaldehyde

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In order to investigate the biological occurrence and function of hydroxypyruvate, suitably sensitive methods for its determination are essential. Hitherto the only qualitative colorimetric reaction described for this substance has been the purple colour with alkaline ferric salts (Fenton & Jones, 1900), but this proved unsuitable for quantitative purposes and similar colours are given by other hydroxyl compounds. The lack of a stable solid preparation of the acid or its salts, for use as an analytical standard, has been overcome by the preparation of the monohydrates of lithium hydroxypyruvate and hydroxypyruvic acid (Dickens & Williamson, 1958). The analytically pure recrystallized lithium salt, $CH_2(OH)\cdot C(OH)_2\cdot CO_2Li$, provides the most suitable standard. By the use of this material a satisfactory colorimetric assay has been developed, based upon the grass-green colour produced on heating hydroxypyruvate solutions with naphtharesorcinol in 23N-sulphuric acid (Dickens & Williamson, 1956*a*). Glycolaldehyde gives the same colour, but may be distinguished by its non-absorption on a mixed-bed resin, as is described below.

A specific enzymic reaction of hydroxypyruvate, which like the above reaction clearly distinguishes it from pyruvate, has been described by Stafford, Magaldi & Vennesland (1954). In the presence of a hydroxypyruvate reductase extracted from parsley leaves, reduction of hydroxypyruvate by reduced diphosphopyridine nucleotide (DPNH) can be followed photometrically. This stoicheiometric reaction has been used to check the purity of our preparations of hydroxypyruvate (Dickens & Williamson, 1958), and its application to determinations in biological material is described below.

In solutions containing both pyruvate and hydroxypyruvate, the total keto acid present may be determined by the oxidation of DPNH in the presence of L-lactic dehydrogenase of muscle, since this enzyme reacts equally well with both substrates (Meister, 1952).

A combination of these various methods is therefore capable of wide application to the analysis of various biological systems.

EXPERIMENTAL

Colorimetric assay of hydroxypyruvate and glycolaldehyde

Solutions. Naphtharesorcinol (L. Light and Co. Ltd.) was recrystallized from 20 vol. of benzene, with a little charcoal added. A solution (5 mg./ml.) in ethanol was freshly prepared before use, and usually had a very faint pink colour.

Sulphuric acid solution (27 N) was prepared by cautiously adding 388 ml. of analytical-grade H_2SO_4 (sp.gr. 1·84) to 147 ml. of water.

As standards, mM-solutions of recrystallized lithium hydroxypyruvate (Dickens & Williamson, 1958) and glycolaldehyde (L. Light and Co. Ltd.) were prepared just before use.

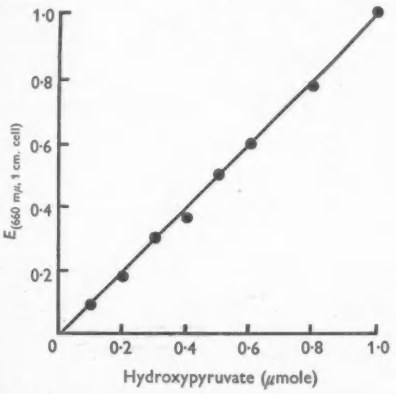

Fig. 1. Calibration curve at 660 mμ of naphtharesorcinol reaction for hydroxypyruvate. The curve for glycolaldehyde is superimposable. The amounts shown are μmoles of lithium hydroxypyruvate in the standard test.

Table 1. *Analysis of mixtures containing hydroxypyruvate and glycolaldehyde*

The quantities are μmoles/5 ml. of solution, weighed as lithium hydroxypyruvate monohydrate and glycolaldehyde respectively. Glycolaldehyde was determined by colorimetric estimation of the portion not adsorbed on mixed-bed resin and hydroxypyruvate by difference, colorimetry on the mixture being used for total μmoles of both substances present (see text).

Amount taken (μmoles)		Found (μmoles)	
Hydroxypyruvate	Glycolaldehyde	Hydroxypyruvate	Glycolaldehyde
2·5	1·0	2·6	0·9
2·5	2·5	2·6	2·6
2·0	2·0	1·9	2·1
1·0	1·0	1·0	0·9

Procedure. The solutions containing hydroxypyruvate (1 ml. containing 0·1–1·0 μmole) in glass-stoppered test tubes are mixed with 0·1 ml. of naphtharesorcinol reagent. A reagent blank (1 ml. of water) and suitable standards (1 ml.) are also similarly treated. The H_2SO_4 reagent (6 ml.) is slowly added to each tube and the contents are well mixed by shaking the stoppered tubes, which are then immersed in a boiling-water bath for 20 min. They are cooled in running water and the density at 660 mμ is read in a spectrophotometer (1 cm. cells). Unless the colour is a clear green the estimation should be rejected, as the presence of interfering substances (see below) is indicated.

With biological material, deproteinization with trichloroacetic acid (3%, w/v) is an essential preliminary, the acidic filtrate being used directly without neutralization. The method cannot be applied to solutions containing much free carbohydrate.

A typical calibration curve is shown in Fig. 1. That obtained for glycolaldehyde, in the same molar amounts, is superimposable on this graph.

Determination of glycolaldehyde and hydroxypyruvate when both are present. The formation of a green colour immediately on mixing with the H_2SO_4 reagent in the above test, before heating, indicates the probable presence of free glycolaldehyde. The final colour developed after heating for 20 min. then gives the total amount (μmoles) of hydroxypyruvate and glycolaldehyde present.

In order to determine the relative amounts of each substance, the hydroxypyruvate is adsorbed by shaking the solution with the mixed-bed ion-exchange resin Bio-Deminrolit G (The Permutit Co. Ltd.) previously found (Dickens & Williamson, 1956*b*) to give maximum recovery of neutral reducing sugars. This has the further advantage that the resulting deionized solutions are excellent for use in paper chromatography (cf. Dickens & Williamson, 1958).

Procedure. The acidic solution (*A*) containing hydroxypyruvate and glycolaldehyde (0·5–10 μmoles of each, in 5 ml.) is shaken in a stoppered test tube with 2 g. of Bio-Deminrolit G until the pH of the liquid, tested with indicator paper, reaches approx. 4. The resin is removed by filtration (filtrate = solution *B*) with slight air pressure on a small sintered-glass disk (porosity 1, 1 cm. diam. sealed into a 10 cm. glass tube). The naphtharesorcinol reaction is carried out on solutions *A* and *B*, as already described.

In practice, whereas the hydroxypyruvic acid is nearly quantitatively absorbed by the resin (99% of 50, 98% of 10 and 95% of 2·5 μmoles), the recovery of glycolaldehyde is consistently between 76 and 83%, mean 80%. Consequently the values found for glycolaldehyde by this method require to be multiplied by a factor of 1·25. Table 1 shows the resulting analysis of the mixed substances.

Notes on the colorimetric method

Absorption spectra of the colorimetric solutions. The absorption spectrum given by hydroxypyruvate is shown in Fig. 2. That given by glycolaldehyde is superimposable, within the experimental limits, suggesting that the same chromogen is concerned. The well-defined absorption maximum is at 660 mμ.

Strength of sulphuric acid used (differential analysis of hydroxypyruvate and glycerate). The strength of acid used affects both the specificity of the method

and the colour developed. In the test described above, the final solution is 23 N in H_2SO_4. When nearly concentrated (35 N) sulphuric acid is used instead, with the minimum practical volumes of aqueous solution and of naphtharesorcinol reagent, only a faint yellow–green is given by hydroxypyruvate. These are approximately the conditions, however, of the Rapoport (1936) method for estimation of glyceric acid, which gives a clear prussian-blue colour suitable for colorimetric estimation. (The specificity of the Rapoport reaction is discussed by Feigl, 1954.)

In 23 N-H_2SO_4, however, with naphtharesorcinol under the standard conditions already described, glyceric acid (10 μmoles) gives zero density at 660 mμ, no trace of blue or green colour being formed. Hence, by simultaneous determinations of the colour given with naphtharesorcinol in nearly concentrated and in 23 N-H_2SO_4, the differential analysis of glycerate and hydroxypyruvate is possible. This principle has been successfully used to follow the enzymic transformation of hydroxypyruvate into glycerate by means of cytochrome b_2 of yeast (Dickens & Williamson, 1956*a*). A period of heating at 100° for 1 hr. is suitable for the estimation of glyceric acid, whereas 20 min. is sufficient for hydroxypyruvate.

The effect of the normality of H_2SO_4 in the heated reaction mixture (containing hydroxypyruvate and naphtharesorcinol) on the subsequent light absorption at 660 mμ is shown in Fig. 3. The optimum is 23 N, final concentration.

It is worth noting that the concentration of the H_2SO_4 reagent (27 N) happens to be the same as that used in the cysteine–carbazole reaction of Dische & Borenfreund (1951) for ketopentoses, and will serve for both estimations.

Period of heating. The rate of colour development at 100° is such that hydroxypyruvate yields maximum colour development in 15 min. and this is unchanged after 20 min., the standard period adopted. After heating for 3 min., only 10 % of this maximum occurs with hydroxypyruvate, whereas 50 % of the maximum is given in this short period by glycolaldehyde. The difference can be increased by using a 70° colour development (Fig. 4), but is still not really adequate for the accurate differential determination of the two substances.

Specificity. A variety of colours is given by a number of substances when these are tested by the standard procedure (Table 2). In the presence of those substances giving strong colours, the method cannot be applied. Unless purification can be achieved, the enzymic-assay method must be used in such cases. Fortunately, the only substance tested other than hydroxypyruvate and glycolaldehyde, which was found to give a clear-green colour, was dihydroxyfumaric (dihydroxymaleic)

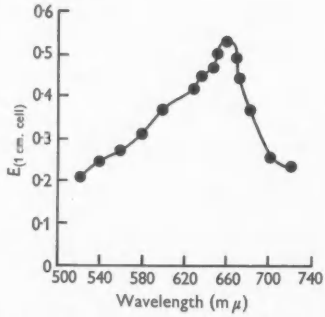

Fig. 2. Absorption spectrum of naphtharesorcinol reaction for hydroxypyruvate. That for glycolaldehyde is superimposable. The amount of either substance taken in the standard test is 0·5 μmole. Peak absorption is at 660 mμ.

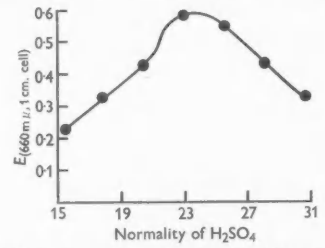

Fig. 3. Effect of normality of sulphuric acid in the final reaction mixture on naphtharesorcinol test for hydroxypyruvate. Lithium hydroxypyruvate (0·5 μmole) was used in conditions as in the standard test, except for the varying concentrations of sulphuric acid.

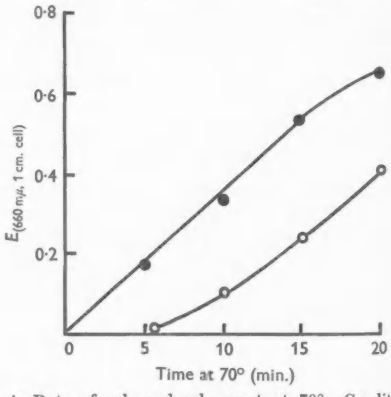

Fig. 4. Rate of colour development at 70°. Conditions were as in the standard naphtharesorcinol test, except for temperature (70° instead of 100°) and periods of heating as shown. ○, Hydroxypyruvate (0·5 μmole); ●, glycolaldehyde (0·5 μmole).

Table 2. *Specificity of the naphtharesorcinol reaction*

All the compounds were tested under standard conditions of the method as described. Sources of material: A, authors' preparation; B, British Drug Houses Ltd., Poole, Dorset; R, Roche Products Ltd., 15 Manchester Square, London, W. 1; Sch, Schering A.G., Berlin; Si, Sigma Chemical Company, St Louis, Mo., U.S.A. The phosphohydroxypyruvic acid was a gift from Dr C. E. Ballou provided through the kindness of Dr D. D. Davies.

Compound	Amount (μmoles)	Final colour	$D\frac{1\,cm.}{660}$	Source
Xylose	1	Cherry-red	0·310	R
Xylulose	1	Faint pink	0·110	A
Ribose	1	Strong violet	0·400	R
Ribulose	1	Brownish red	0·300	A
Erythrose	2	Grey–green	0·190	A
Erythrulose	2	Grey–green	0·195	A
Glyceric acid	2	None	0·000	A
Glycollic acid	2	None	0·000	B
Glyceraldehyde	2	Strong grey	0·980	Sch
Fructose	2	Brown with greenish fluorescence	0·780	B
Glucose	2	Brown with greenish fluorescence	0·740	B
Glucuronic acid	2	Brownish red	0·570	Si
Ascorbic acid	1	Faint pink	0·075	B
Pyruvic acid	10	Faint yellow	0·012	R
Glyoxylic acid	2	Orange–brown	0·150	A
Formaldehyde	1	Olive–brown	0·125	B
Dihydroxymaleic (dihydroxyfumaric) acid	0·5	Green	0·290	A
Glycolaldehyde	0·5	Strong green	0·610	A
Hydroxypyruvic acid	0·5	Strong green	0·600	A
Phosphohydroxypyruvic acid	0·5	Strong green	0·580	Gift

acid. It is probable that on heating with H_2SO_4 of this strength both acids are decarboxylated to give glycolaldehyde.

Glyoxylic acid shows a well-defined maximum absorption at 480 mμ in the standard test (1 μmole gives a density of 0·8 at 480 mμ compared with only 0·1 at 660 mμ). Consequently either glyoxylic or hydroxypyruvic acid may be estimated by comparison of the densities at these two wavelengths, provided that the other substance is not present in large excess.

Enzymic assay of hydroxypyruvate

The colorimetric method is quick and convenient, but is applicable only when the amounts of interfering substances present are inconsiderable. If this is not so, and also where high specificity is needed, the photometric estimation of reoxidation of DPNH by means of the hydroxypyruvate reductase of Stafford *et al.* (1954) is preferable. This enzyme causes the reduction of hydroxypyruvate to glycerate:

$$CH_2(OH)\cdot C(OH)_2\cdot CO_2^- + DPNH + H^+ \rightarrow CH_2(OH)\cdot CH(OH)\cdot CO_2^- + DPN^+ + H_2O.$$

The reaction is virtually irreversible except at alkaline pH with high glycerate concentration. Although Sallach (1956) has recently used this reaction to measure changes in hydroxypyruvate in the L-serine–L-alanine transamination system, no detailed description of the assay of hydroxypyruvate by this useful method appears to have been published hitherto.

Materials. Diphosphopyridine nucleotide (enzymically reduced form, 85% DPNH), was purchased (Boehringer und Soehne, Mannheim). A 0·5% solution (10 ml.) is adjusted to pH 8 with dilute NaOH and stored at −15°.

Phosphate buffer, 0·1 M, pH 7·2, is prepared by mixing 3 vol. of 0·1 M-KH_2PO_4 with 7 vol. of 0·1 M-K_2HPO_4.

Hydroxypyruvate reductase (cf. Stafford *et al.* 1954). Parsley leaves are washed, frozen at −15° overnight and minced in a domestic mincer. The juice is expressed (40 ml. from 100 g. of mince), and centrifuged. To the decanted clear fluid (30 ml.) saturated $(NH_4)_2SO_4$ soln. (45 ml.) is added. After several hours at 0° the precipitate is collected and the precipitation with $(NH_4)_2SO_4$ is repeated. Finally, the precipitate is dissolved in 12 ml. of 0·01 M-phosphate, pH 7·4. Stored at −15°, the solution remains active for months.

Method. Solutions for analysis are first deproteinized, when necessary, by trichloroacetic or perchloric acid, followed by cautious neutralization with dilute alkali.

Into a 1 cm. silica cell are measured 1 ml. of phosphate buffer, 0·1 ml. of DPNH, the test solution (or hydroxypyruvate, 0·05–0·4 μmole, as standard), and water to make 3 ml. total volume. Two other cells are simultaneously set up, one a control with all reagents except hydroxypyruvate and the other a blank with water only (3 ml.). The control and hydroxypyruvate densities are read against the blank at 340 mμ in a Unicam SP. 600 spectrophotometer. Then to each of the three cells hydroxypyruvate reductase (0·02 ml.) is added at zero time. Readings are taken at minute intervals until the rate of decrease of density becomes very slow and equal in the cells with and without hydroxypyruvate (7–10 min.). The total density decrease of the control is subtracted from that of the hydroxypyruvate cell. This value × 0·478 gives the μmoles of hydroxypyruvate present in the cell; this factor is calculated for an extinction coefficient of $6{\cdot}3 \times 10^6$ cm.^{2}/mole for DPNH.

Table 3. *Determination of hydroxypyruvate by enzymic oxidation of diphosphopyridine nucleotide with hydroxypyruvate reductase*

Amounts are μmoles of hydroxypyruvate, weighed as the lithium salt monohydrate, contained in 3·02 ml. in 1 cm. spectrophotometer cells. For details see text.

Amount taken (μmole)	Found (μmole)	Recovery (%)
0·10	0·095	95·5
0·20	0·195	98
0·30	0·308	102·5
0·40	0·385	97
	Mean	98

Notes on the enzymic method

Specificity. Pyruvate is reduced at less than 2 % of the rate of an equimolar amount of hydroxypyruvate by this preparation of the enzyme. Since both pyruvate and hydroxypyruvate rapidly oxidize DPNH in the presence of lactic dehydrogenase of muscle, which is obtainable commercially (Boehringer und Soehne, Mannheim), a differential analysis of pyruvate and hydroxypyruvate in mixtures of the two substances is readily performed by a comparison of the density decrease obtained with these two enzymes either separately or consecutively in the same solution.

Recovery of hydroxypyruvate. When recrystallized lithium hyproxypyruvate monohydrate (Dickens & Williamson, 1958) was used as a standard, the recovery was quantitative within the experimental limits of ± 5 % (Table 3).

SUMMARY

1. A colorimetric method for the determination of hydroxypyruvate and glycolaldehyde is described. The intensity of the green colour obtained on heating the solution with naphtharesorcinol in 23 N-H_2SO_4, measured at 660 mμ, is proportional to the amount of these substances present under the defined conditions of the test.

2. When both substances are present together the estimation is repeated after adsorption of hydroxypyruvic acid on Bio-Deminrolit G.

3. The specificity of the test has been studied, and certain interfering substances are noted. Modifications permitting the estimation of glyceric acid and of glyoxylic acid are indicated.

4. The use of hydroxypyruvate reductase from parsley leaves and lactic dehydrogenase of muscle for enzymic assay of hydroxypyruvate is described. Whereas the former enzyme reacts only with hydroxypyruvate, the latter reduces pyruvate also and the two enzymes may therefore be used in conjunction for quantitative analysis of mixtures of these two keto acids.

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The Degradation of Cartilage Chondroitin Sulphate by the Chondroitinase of *Proteus vulgaris*

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Dodgson, Lloyd & Spencer (1957) have shown a strain of *Proteus vulgaris* (National Collection of Type Cultures, no. 4636) to be a particularly potent source of two enzymes, chondroitinase and chondrosulphatase, which are collectively capable of degrading cartilage chondroitin sulphate with release of reducing substances and sulphuric acid respectively. Subsequent work (Dodgson & Lloyd, 1957*a*, *b*) showed that whereas chondroitinase action could proceed independently of the associated chondrosulphatase, the latter enzyme was inactive towards polymer chondroitin sulphate. However, the enzyme was capable of liberating sulphuric acid from the sulphated oligosaccharide fragments which were obtained by degrading cartilage chondroitin sulphate with testicular

hyaluronidase. The present communication describes the isolation and characterization of the sulphated disaccharide resulting from the exhaustive degradation of cartilage chondroitin sulphate by *Proteus* chondroitinase in the absence of chondrosulphatase action. A preliminary report of this work has already been made (Lloyd & Dodgson, 1957).

MATERIALS AND METHODS

Potassium chondroitin sulphate. Three different types of chondroitin sulphate are known to occur in mammalian tissues (Meyer & Rapport, 1951; Orr, 1954; Meyer, Davidson, Linker & Hoffman, 1956). Two of these types, chondroitin sulphates A and C, are present in cartilage, the former predominating. The third type, chondroitin sulphate B, occurs mainly in skin, tendon and heart-valves but is not present in cartilage (Meyer *et al.* 1956). Further comment regarding the differences between the three types is made in a later section. The chondroitin sulphate used in the present work was prepared from fresh bovine tracheal cartilage by the method described by Dodgson *et al.* (1957) and may be presumed to consist mainly of chondroitin sulphate A together with small amounts of the C form.

Chondrosin and N-acetylchondrosin. Chondrosin, ?-*O*-(β-D-glucopyranosyluronic acid)-2-deoxy-2-amino-D-galactose, was prepared by the method of Davidson & Meyer (1954) and was subsequently converted into the corresponding *N*-acetyl derivative by treatment with keten, the procedure described by Weissmann & Meyer (1954) for the preparation of *N*-acetylhyalobiuronic acid being used. Davidson & Meyer (1954) have shown that in chondrosin the uronic acid is linked to the galactosamine residue through the reducing group of the former. However, the actual position of linkage has not yet been established with certainty. Similar considerations therefore apply to *N*-acetylchondrosin and to the *N*-acetylchondrosin sulphate described in a later section. In addition, the position of the sulphate group in the latter compound is not known.

Enzyme preparations and methods of assay. Bovine testicular hyaluronidase was prepared by the method of Dorfman (1955). The preparation of concentrates of chondroitinase and chondrosulphatase from extracts of *Proteus vulgaris* has already been described (preparations *C*2 and *D* respectively, see Dodgson & Lloyd, 1957*b*). Methods for the assay of hyaluronidase and chondroitinase (based on determination of release of reducing substances) and for chondrosulphatase (based on determination of liberated SO_4^{2-} ions) have already been described (Dodgson *et al.* 1957).

Analytical methods. Total nitrogen was determined by the method of Markham (1942), uronic acid by the carbazole method of Dische (1947) and amino nitrogen by the method of Moore & Stein (1948). The hexosamine and ester sulphate contents of various compounds were determined by the methods of Belcher, Nutten & Sambrook (1954) and Dodgson & Spencer (1953) respectively, after hydrolysis of the compounds with 4N-HCl for 15 hr. at 100°.

Descending paper chromatography. This was carried out on Whatman no. 1 paper. The solvent system used was butan-1-ol–acetic acid–water, either in the proportions 50:12:25 or 50:15:35. Hexosamine-containing oligosaccharides were detected on chromatograms with Elson–Morgan reagents (see Partridge, 1948), compounds containing free amino groups with a solution of ninhydrin in butan-1-ol (Payne & Kieber, 1954) and materials possessing general reducing properties by using a solution of aniline hydrogen phthalate in *n*-butanol-saturated water (Partridge, 1949).

Charcoal-column chromatography. Enzyme digests of chondroitin sulphate were freed from enzyme and electrolyte by chromatography on columns consisting of a mixture (1:1) of Darco G-60 charcoal (Atlas Powder Co., New York, U.S.A.) and Celite no. 535 (Johns Manville Co., New York, U.S.A.). The dimensions of the columns were adjusted according to the concentration of chondroitin sulphate originally present in the enzyme digest, 3 g. of the adsorbent mixture being used for each 100 mg. of chondroitin sulphate. Before use, columns were washed for several hours with water, followed by 50% (v/v) ethanol and finally with water again, flow rates being adjusted to approx. 2 ml./min. by application of air under pressure.

EXPERIMENTAL AND RESULTS

Time-course of the degradation of chondroitin sulphate by Proteus *chondroitinase*

It had previously been established (Dodgson & Lloyd, 1957*b*) that *Proteus* chondroitinase could be studied independently of the associated chondrosulphatase by taking advantage of the fact that the latter enzyme could be completely inhibited by 0·1M-Na_3PO_4 without affecting chondroitinase activity. In practice the required amount of the enzyme preparation containing both chondroitinase and chondrosulphatase (preparation *C*2, see Dodgson & Lloyd, 1957*b*) was dissolved in a known volume of 0·2M-sodium acetate–acetic acid mixture, pH 7·5, before adding an equal volume of a 0·2M-solution of Na_3PO_4. The pH of the mixture was re-adjusted to 7·5 with acetic acid and the whole incubated at 37·5° for 30 min. The treated enzyme preparation exhibited no sulphatase activity towards chondroitin sulphate or the sulphated oligosaccharides arising from the exhaustive degradation of chondroitin sulphate by testicular hyaluronidase (cf. Dodgson & Lloyd, 1957*b*). The inhibition could not be reversed by dialysis of the treated preparation. In all experiments involving chondroitinase preparation *C*2 which had been treated in this way was used.

When examining the degradation of chondroitin sulphate by testicular hyaluronidase a similar mixture of sodium acetate, acetic acid and Na_3PO_4 was used as buffer at pH 4·5. This ensured that both chondroitinase and hyaluronidase experiments were performed in a similar electrolyte environment. When treated as described above chondroitinase showed maximum activity towards chondroitin sulphate (1%, w/v solution, final concentration) at pH 7·8 when assayed over a period of 1 hr. at 37·5°. Under similar conditions testicular

hyaluronidase showed maximum activity at pH 4·5.

Fig. 1 shows the time-courses of the degradation of chondroitin sulphate by chondroitinase and testicular hyaluronidase respectively. In each case 10 ml. of a 1% (w/v) solution of enzyme in the acetate–phosphate buffer described above and at the pH appropriate to the enzyme was incubated at 37° with an equal volume of a 2% (w/v) solution of chondroitin sulphate in the same buffer. Samples were withdrawn at suitable time intervals and assayed for release of reducing substance. It can be seen from Fig. 1 that the release of reducing substance proceeded more rapidly with chondroitinase than with testicular hyaluronidase, the reaction being complete in 24 hr. with the former enzyme, since no further increase in reducing activity occurred on addition of a further 3 ml. of freshly prepared enzyme solution at the twenty-eighth hour. Paper chromatography of the material incubated with chondroitinase, with the solvent systems described earlier, showed the presence of one carbohydrate component only ($R_p = 0{\cdot}17$ and 0·22 in the solvent systems 50:12:25 and 50:15:35 respectively). The component gave positive reactions for reducing groups and hexosamine but a negative ninhydrin reaction, indicating the absence of free amino groupings. Paper chromatography of the solutions incubated with hyaluronidase under similar conditions showed the presence of three carbohydrate components containing reducing groups and hexosamine but no free amino groups. In addition, some material remained at the starting point. None of these components was as mobile as the chondroitinase product. However, if the hyaluronidase incubation period was prolonged to 48 hr. a faint spot corresponding to this product was obtained (see Fig. 2). Further comment on these points will be made later. In all cases control experiments were made in which enzyme preparation and substrate were incubated separately and chromatographed under the conditions outlined above. No mobile components were observed.

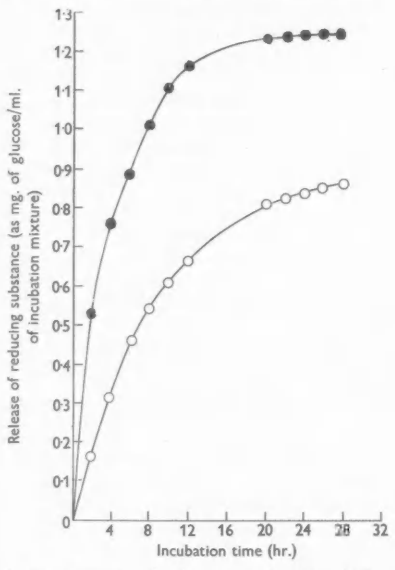

Fig. 1. Time-course of the enzymic degradation of cartilage potassium chondroitin sulphate. Experimental conditions are described in the text. ●, Chondroitinase of *Proteus vulgaris*; ○, testicular hyaluronidase.

Isolation of the product of chondroitinase action

Preliminary experiments were conducted in order to establish the conditions necessary for the separation of the product of chondroitinase action from the other components of the incubation mixture. In a typical experiment 15 ml. of a 1% (w/v) solution of chondroitinase in the acetate–phosphate mixture described earlier was incubated for 24 hr. at 37° and pH 7·8 with an equal volume of a 2%

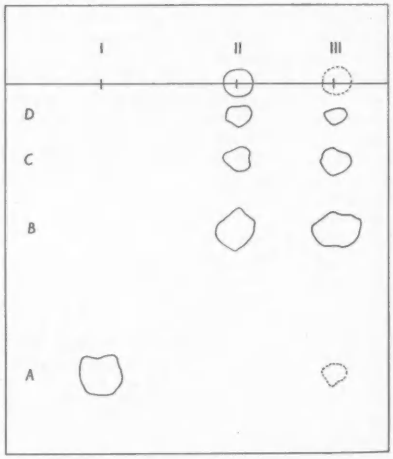

Fig. 2. Paper chromatography of the products of the enzymic degradation of cartilage potassium chondroitin sulphate. I, Degradation for 24 hr. with *Proteus* chondroitinase; II and III, degradation for 24 and 48 hr. respectively with testicular hyaluronidase. R_p values in the solvent system butan-1-ol–acetic acid–water (50:12:25) were: *A*, 0·17 (0·22); *B*, 0·079 (0·19); *C*, 0·037 (0·12); *D*, 0·01 (0·08). Figures in parentheses are the values obtained with the same solvent system in the proportions 50:15:35.

(w/v) solution of chondroitin sulphate in the same acetate–phosphate mixture. The incubation mixture was subsequently transferred to a charcoal column (prepared as described above) which was then washed with water until PO_4^{3-} ions could no longer be detected in the eluate. Elution of oligosaccharide material was then attempted by successive washings with 5 and 15% ethanol followed by 0·1, 0·5, 1, 1·5, 2 and 5% pyridine (all percentages being v/v). Eluates were collected automatically in 10 ml. fractions, which were screened for uronic acid by the method of Dische (1947), a new solvent being introduced when the eluates no longer contained uronic acid. Most of the uronic acid-containing material was present in the ethanol eluates but a small amount was present in the 0·1% pyridine eluate. These eluates were concentrated separately by distillation *in vacuo* at 40° and examined by paper chromatography. Although the elution pattern obtained suggested that separation of three distinct components had been achieved, in actual fact each eluate contained the same single component, the chromatographic mobility of which was identical with that of the component originally demonstrated by chromatography of the whole incubation mixture. A similar phenomenon was observed by Linker, Meyer & Hoffman (1956) during the chromatographic separation (charcoal–Celite column) of the unsaturated uronide resulting from the degradation of hyaluronic acid by bacterial hyaluronidase.

Isolation of the degradation product was carried out on a larger scale as follows. The incubation mixture consisted of 1 g. of chondroitinase and 2 g. of chondroitin sulphate in a total volume of 200 ml. of the usual acetate–phosphate buffer at pH 7·8. After incubation for 24 hr. at 37° the mixture was transferred to a charcoal column which was subsequently washed with water in order to remove electrolyte. The column was then eluted in the manner already described, the eluates being collected automatically in 25 ml. fractions which were screened for uronic acid. Fig. 3 shows the elution pattern obtained. Peak materials were concentrated to low bulk separately, by distillation *in vacuo* at 40°, and examined by paper chromatography. Peaks 1–4 (see Fig. 3) corresponded to the single component already described. The remaining peaks (5–7) represented three further, poorly resolved, components of lower chromatographic mobilities. Mention will be made of these components later.

The concentrates corresponding to peaks 1–4 were combined, reduced to low bulk by distillation *in vacuo* at 40° and passed through a Dowex 50 ion-exchange column (17 cm. × 2 cm., K^+ form, 50–100 mesh; Dow Chemical Co., Michigan, U.S.A.). The eluate and water-washings from the column were combined, concentrated to low bulk by distillation *in vacuo* at 40° and finally freeze-dried. The product was a pale-yellow powder; yield, 520 mg.

Characterization of the product of chondroitinase action. The product was chromatographically homogeneous and gave positive tests for hexosamine and reducing groups but no ninhydrin test for free amino groups. The compound had $[\alpha]_D^{20} - 14 \pm 2°$ in water ($c = 2$) (Found: N, 2·5; amino N, 2·5; ester SO_4^{2-} ions, 17·8; uronic acid 35·8; hexosamine, 33·2. The dipotassium salt of *N-acetylchondrosin sulphate* (new compound), $C_{14}H_{21}O_{15}NSK_2$, requires N, 2·5; amino N, 2·5; ester SO_4^{2-} ions, 17·4; uronic acid, 35·0; hexosamine, 32·4%). There was no evidence of the presence of an unsaturated linkage in the uronic acid moiety (cf. the unsaturated uronide resulting from the action of bacterial hyaluronidases on hyaluronic acid; Linker *et al.* 1956) since the compound failed to take up bromine and had no characteristic ultraviolet-absorption peak at 232 mμ. Hydrolysis of the compound and subsequent treatment of the hydrolysate under the conditions outlined by Davidson & Meyer (1954) for the preparation of chondrosin from chondroitin sulphate, yielded material which moved as a single spot on paper chromatography with a mobility identical with that of chondrosin ($R_F = 0·13$ in the solvent system 50:12:25).

Further evidence as to the nature of the oligosaccharide was obtained as follows. The oligosaccharide (75 mg. in 2 ml. of 0·2 M-sodium acetate–acetic acid mixture, pH 7) was incubated with

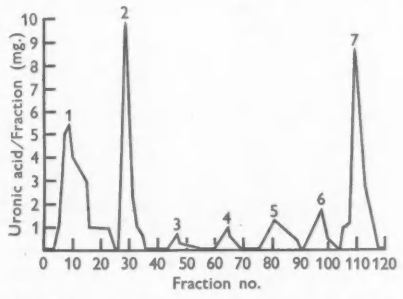

Fig. 3. Elution pattern resulting from the charcoal-column chromatography of the products of the degradation of chondroitin sulphate by the chondroitinase of *Proteus vulgaris*. Experimental points were obtained for each fraction (25 ml.) but have been omitted for the sake of clarity. Elution solvents were: fractions 1–25, 5% ethanol; 26–40, 15% ethanol; 41–56, 0·1% pyridine; 57–68, 0·5% pyridine; 69–76, 1% pyridine; 77–90, 1·5% pyridine; 91–102, 2% pyridine; 103–120, 5% pyridine.

3 ml. of a 1·2% solution of chondrosulphatase in the same acetate mixture for 3 hr. at 37°. No increase in reducing activity occurred during this treatment. The incubation mixture was transferred to a charcoal column (12 cm. × 1 cm.) which was subsequently washed with water (200 ml.) followed by 5% (v/v) pyridine (200 ml.). The pyridine eluate was concentrated to dryness *in vacuo* at 35°, the residue dissolved in water and examined by paper chromatography together with samples of the original starting material, chondrosin and *N*-acetylchondrosin and with the solvent system 50:12:25. The component resulting from the action of chondrosulphatase on the unknown oligosaccharide had a chromatographic mobility identical with that of *N*-acetylchondrosin ($R_F = 0{\cdot}32$). A small amount of the starting material ($R_F = 0{\cdot}17$) remained unchanged. It seems clear from the collective results that the ultimate product of the degradation of chondroitin sulphate by *Proteus* chondroitinase is *N*-acetylchondrosin sulphate.

Incomplete degradation of chondroitin sulphate by Proteus *chondroitinase*

Examination of the charcoal-column eluates after the large-scale degradation of chondroitin sulphate by chondroitinase showed that, in addition to *N*-acetylchondrosin sulphate, other oligosaccharides were also produced and appeared in the 1·5, 2 and 5% pyridine eluates (see Fig. 3, peaks 5–7). It was possible that these materials represented intermediate products in the degradation of chondroitin sulphate. Further experiments were therefore designed in order to establish the nature of the products of the incomplete degradation of chondroitin sulphate.

Chondroitinase (60 mg.) was incubated, for 15 hr. only, with chondroitin sulphate (300 mg.) in a total volume of 30 ml. under the conditions described earlier. The incubation mixture was transferred to a charcoal column which was subsequently washed with water until no further PO_4^{3-} ions could be detected in the eluate and then eluted with 5% (v/v) pyridine. Fractions containing uronic acid were combined, concentrated to low bulk *in vacuo* at 40° and finally freeze-dried. Paper chromatography under the conditions described previously showed the presence of four components in the freeze-dried material. All the components gave positive tests for hexosamine and general reducing groups but no ninhydrin test for free amino groups. The principal component had a chromatographic mobility which was identical with that of *N*-acetylchondrosin sulphate, whereas the remaining components exhibited a pattern of decreasing mobility. The chromatographic pattern as a whole was identical with that obtained when chondroitin sulphate was degraded with testicular hyaluronidase for a period of 48 hr. (see Fig. 2), and it must therefore be assumed that the same oligosaccharide series is obtained in each case.

French & Knapp (1950) have observed empirically that the log of the partition function

$$[\log (1/R_F) - 1]$$

is an additive property of the various structural features of a given oligosaccharide molecule. Moreover, it is related to the degree of polymerization of that molecule and, to some extent, to the type of linkage involved in the formation of the molecule. These workers showed that a plot of the log of the partition function against the degree of polymerization for a homologous series of oligosaccharides yielded a straight line. Since these early observations this principle has been successfully applied to a number of different known homologous series of glucose and fructose oligosaccharides (e.g. French, 1951; French & Wild, 1952, 1953; White & Secor, 1953; Feingold, Avigad & Hestrin, 1956) and to the oligosaccharides

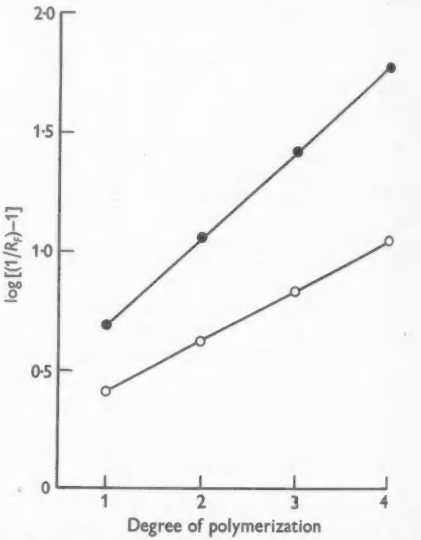

Fig. 4. Relationship between assumed degree of polymerization and paper-chromatographic mobilities of the oligosaccharide series resulting from the incomplete degradation of chondroitin sulphate by *Proteus* chondroitinase or the 48 hr. degradation by testicular hyaluronidase. The polymer repeating unit is assumed to be *N*-acetylchondrosin sulphate. ●, Solvent system butan-1-ol–acetic acid–water in the proportions 50:12:25; ○, the same solvent system in the proportions 50:15:35.

resulting from the degradation of hyaluronic acid by testicular and bacterial enzymes (Weissmann, Meyer, Sampson & Linker, 1954; Schütte & Greiling, 1955).

Examination of the chromatographic behaviour of the oligosaccharide series resulting from the incomplete degradation of chondroitin sulphate by *Proteus* chondroitinase or from the 48 hr. degradation by testicular hyaluronidase showed that this series also obeys this principle. If it is assumed that *N*-acetylchondrosin sulphate is the repeating unit of each oligosaccharide and that the degree of polymerization has whole-number values, then a linear relationship exists between the degree of polymerization and the log of the partition function (Fig. 4). It would therefore appear that these oligosaccharides consitute a homologous series, namely di-, tetra-, hexa- and octa-saccharides, and that the action of testicular hyaluronidase towards chondroitin sulphate is essentially similar to that of chondroitinase, although with regard to the preparations used in the present work the latter enzyme is the more efficient of the two.

DISCUSSION

On the basis of their action towards hyaluronic acid and chondroitin sulphate two types of aminopolysaccharase systems have previously been differentiated. That of mammalian origin, testicular hyaluronidase, degrades both aminopolysaccharides to give mainly tetrasaccharide products, although disaccharides and higher oligosaccharides may also be formed in small amounts (Weissmann *et al.* 1954; Schütte & Greiling, 1955; Hoffman, Meyer & Linker, 1956). Evidence indicates that the products of testicular hyaluronidase action have the same repeating units as the original polymers. In contrast, the hyaluronidases of bacterial origin degraded hyaluronic acid to yield mainly a disaccharide, although higher oligosaccharides may also be produced (Schütte & Greiling, 1955; Linker *et al.* 1956). This disaccharide is not identical with the repeating unit of the original polymer and has been identified as the unsaturated uronide 3-*O*-(β-D-Δ-3:4-glucoseenpyranosyluronic acid)-*N*-acetyl-2-deoxy-2-amino-D-glucose (Linker *et al.* 1956). Chondroitin sulphate A is not attacked by these bacterial hyaluronidases (Meyer & Rapport, 1951) unless ester sulphate groupings are first removed by mild acid hydrolysis (Linker *et al.* 1956). The action of bacterial hyaluronidases on the desulphated, partially degraded product yields an unsaturated disaccharide similar to that obtained from hyaluronic acid, although its structure does not appear to have been established with certainty (Linker *et al.* 1956).

Little is known of the effects of testicular and bacterial hyaluronidases on chondroitin sulphates B and C. Preliminary reports (Meyer & Rapport, 1951) indicate that neither compound is attacked by the bacterial enzymes. Testicular hyaluronidase is able to degrade chondroitin sulphate C but is without action on the B form (Meyer *et al.* 1956).

It is clear from the present work that *Proteus* chondroitinase degrades cartilage chondroitin sulphate in a manner analogous to that of testicular hyaluronidase, although the disaccharide *N*-acetylchondrosin sulphate, rather than the corresponding tetrasaccharide, is the predominating product. We are thus provided with the first example of a bacterial aminopolysaccharase which is analogous in action to the mammalian enzyme. It has already been pointed out that cartilage chondroitin sulphate consists mainly of chondroitin sulphate A with smaller amounts of the C form. The differences between the two isomers are not great and have been discussed at length by Meyer *et al.* (1956). Both isomers appear to contain the same repeating unit but differ in optical rotation and in their solubility in mixtures of calcium acetate buffer and ethanol. On the other hand, it has recently been established that chondroitin sulphate B differs from A and C with respect to the uronic acid moiety, which is now known to be iduronic acid (Cifonelli, Ludoweig & Dorfman, 1957). Since testicular hyaluronidase is without action on chondroitin sulphate B, it will now be interesting to see if similar considerations apply to chondroitinase.

Dodgson & Lloyd (1957*b*) have already shown that chondroitinase-free chondrosulphatase is inactive towards polymerized cartilage chondroitin sulphate but liberates sulphuric acid from the sulphated products arising from the exhaustive degradation of chondroitin sulphate by testicular hyaluronidase. The present work has shown that *N*-acetylchondrosin sulphate is also a substrate for chondrosulphatase. It is now hoped to isolate various sulphated oligosaccharides after the incomplete degradation of chondroitin sulphate by chondroitinase in order to examine the possible relationships between chondrosulphatase activity and increasing degree of polymerization.

SUMMARY

1. The exhaustive degradation of small amounts of cartilage chondroitin sulphate by the chondroitinase of *Proteus vulgaris*, N.C.T.C. no. 4636, yields a single product containing approximately equimolar amounts of uronic acid, ester sulphate and hexosamine.

2. Acid hydrolysis of this product yields chondrosin, whereas enzymic removal of ester sulphate groupings by the chondrosulphatase of

Proteus vulgaris yields *N*-acetylchondrosin. On the bases of these findings, together with analytical results, the product is considered to be *N*-acetylchondrosin sulphate.

3. When chondroitinase action is not allowed to proceed to completion a series of oligosaccharides is obtained, the principal member being identical with the single product obtained by exhaustive degradation. This series of oligosaccharides is identical with that arising from the prolonged degradation (48 hr.) of chondroitin sulphate with testicular hyaluronidase.

4. Evidence suggests that this oligosaccharide series consists of di-, tetra-, hexa- and octa-saccharide homologues, the smallest member being *N*-acetylchondrosin sulphate.

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Electrophoretic Studies on Bacteria

2. THE EFFECT OF ENZYMES ON RESTING SPORES OF *BACILLUS MEGATERIUM*, *B. SUBTILIS* AND *B. CEREUS**

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In a previous paper (Douglas, 1955) the electrophoretic behaviour of resting spores of *Bacillus subtilis* and *Bacillus megaterium* was reported, as a function of pH and ionic strength, together with the effect on the behaviour of addition of surface-active agents. It was concluded that the outermost, electrokinetically effective, surface layers of the *B. subtilis* and *B. megaterium* spores were essentially non-ionogenic, acquiring their charge by the adsorption of ions, and that they were different with respect to either or both of their chemical and physical structures. Comparison with the corresponding behaviour of bacterial cells and of protein, lipid and other surfaces, as recorded in the literature, suggested that the effective surface of *B. subtilis* spores was essentially polysaccharide; for the *B. megaterium* spores the evidence suggested surface lipid, with possibly a protein component in a complex so as to render the normal ionogenic groups inoperative.

* Part 1: Douglas (1955).

Enzymes have been used with considerable success to indicate the presence of particular types of biological polymer in the surface layers of bacterial cells. The enzymes used include lysozyme (Epstein & Chain, 1940; Wiebull, 1953; Salton, 1955), trypsin and lipase (Dyar, 1948), ribonuclease (Harris, 1953). In some of the studies (Dyar, 1948; Harris, 1953) electrophoresis was used as the means of observing changes in surface composition.

In seeking further information concerning the surface composition of resting spores of *B. subtilis*, *B. megaterium* and *Bacillus cereus* therefore, their electrophoretic behaviour before and after treatment with active preparations of a series of enzymes has been determined, essential control experiments being performed. The enzymes used were lysozyme, hyaluronidase, trypsin, ribonuclease, lipase, lecithinase and a glutamylpeptidase.

The present paper reports the results obtained and draws modified conclusions concerning the nature of the spore surfaces, on the basis of these enzyme effects together with mobility–pH results obtained for model particles having adsorbed surface layers of isolated bacterial peptides (Douglas & Shaw, 1957).

EXPERIMENTAL

Spores. The spores used in the present work were from the same strains of *B. subtilis*, *B. megaterium* and *B. cereus* as those employed by Powell and co-workers in a series of biochemical studies (Powell & Strange, 1953; Strange & Powell, 1954; Strange & Dark, 1956). They were supplied as suspensions in sterile water containing about 2×10^{10} spores/ml., and stored as such.

Enzymes. Samples of crystallized lysozyme (egg white), trypsin (bovine pancreas) and ribonuclease (bovine pancreas) were obtained from Armour Chemicals Ltd., Hampden Park, Eastbourne, Sussex. A second sample of lysozyme from L. Light and Co., Colnbrook, Bucks, behaved identically with the sample from Armour Ltd.

An active preparation of hyaluronidase (Hyalase, 1000 units/mg.; Bengers Ltd.) was kindly supplied by Dr E. London, Bengers Ltd.

Lipase in the form of an active, acetone-dried extract from pig pancreas was kindly supplied by Mr R. Strange, Microbiological Research Establishment, Porton, Wilts.

The lecithinase preparation was a filtrate of a glycerol-treated culture of *Clostridium welchii* type A (Macfarlane & Knight, 1941) obtained from M.R.E., Porton.

The glutamylpeptidase (Thorne, 1956) was generously supplied by Dr C. B. Thorne (then at M.R.E., Porton) as an active preparation in aqueous solution.

Enzyme treatment of spores. A brief review of the literature concerning the susceptibility of bacteria to enzymes showed that the concentrations of enzyme and temperature used have varied considerably with the object of the experiments. Thus lysozyme was used at a final concentration of 4 μg./ml. and 34° to study the changes in appearance produced by it in cells of *B. megaterium* (Welshimer, 1953), whereas to isolate proptoplasts of the same organism 0·5 mg./ml. was used at room temperature (Wiebull, 1953).

In the present work 37° has been used as the temperature for the treatments of active enzyme, which have been carried out in phosphate media, $I = 0{\cdot}1$, of appropriate pH. Periods of treatment of 3, 6 and up to 24 hr. have been used with each of a number of enzyme concentrations to determine the change in electrophoretic behaviour produced by each of the enzymes. When a change was found the enzyme concentration was increased until no further mobility shift resulted. The upper concentration limits quoted below exceed those at which limiting shifts were observed with some of the enzymes.

Lysozyme was used in phosphate buffer, pH 6·8, and at final concentrations between 2 and 100 μg./ml. Trypsin and ribonuclease were used at concentrations within the same range and at pH values 8·0 and 6·8 respectively. The hyaluronidase preparation was used at pH 5·9 and at concentrations 50 μg./ml. to 1 mg./ml. The solid lipase preparation as received was assumed 5% active and an amount sufficient to give a final 'active' concentration of 20 μg./ml. extracted by suspension in phosphate buffer, pH 6·8; the insoluble residue was centrifuged off and the supernatant liquid used in the spore treatments. The original active solutions of lecithinase and of glutamylpeptidase were used at final volume dilutions of 10 to 100 times, in phosphate buffers of pH values 6·8 and 8·0 respectively.

The general experimental procedure for the enzyme treatments was as follows. A small volume of stock spore suspension in water was spun down in an angle centrifuge, the supernatant liquid decanted and the spores were resuspended in a sufficient volume of phosphate buffer, $I = 0{\cdot}1$, of pH appropriate to the enzyme being used, to give a number concentration of about 2×10^{10} spores/ml. A volume (1 ml.) of this was diluted to 10 ml. with the same buffer containing the enzyme at the desired concentration, in a 15 ml. centrifuge tube. This was transferred to a water thermostat at 37° for the incubation period, and stirred from time to time with a sterilized glass rod to maintain the spores in suspension. The tube was then removed, the spores were spun down and the supernatant buffer was discarded. The spores were washed three times by resuspending in 0·05 N-NaCl and spinning down. Finally the washed spores were resuspended in 100 ml. of Michaelis buffer, $I = 0{\cdot}05$, to give a spore concentration of about 2×10^8 spores/ml., which was convenient for the electrophoretic observations.

Control experiments. To allow for the possibilities that (i) heating to 37° alone, (ii) the inactivated enzyme preparation, (iii) adsorption of (*a*) the enzyme or (*b*) other components in the enzyme preparations, might be the cause of any shift in electrophoretic behaviour observed after the above treatments, the following control experiments were performed. The possibility that germination might be the cause could be ruled out since phase-contrast microscopy was used to confirm that the spores, on which electrophoretic measurements were made, retained their appearance as resting spores.

The action of (i), incubation alone, was determined by performing experiments under the conditions described in the previous section, but without the addition of the enzyme preparation. Possibility (ii) was taken account of by repeating the experiments as for active enzyme but with inactivated materials. Possibility (iii) was checked by

allowing parallel systems of spores plus enzyme to stand for up to 3 hr. at room temperature. This was to permit adsorption but to minimize any enzyme attack. As in the experiments at 37°, the spores were then centrifuged, washed three times with 0·05 N-NaCl and finally resuspended in Michaelis buffers of pH 3 and 7, $I = 0{\cdot}05$. Their mean electrophoretic mobility was then determined.

Buffer systems. KH_2PO_4–Na_2HPO_4 buffers, $I = 0{\cdot}1$, were used for all the enzyme and control experiments. The electrophoretic measurements, on the other hand, were carried out in Michaelis buffers, $I = 0{\cdot}05$, as in the previous work on resting spores (Douglas, 1955). The buffer NaCl and HCl used were all of A.R. or equivalent quality. Sterile solutions and distilled water were used throughout the work.

Electrophoresis. The determinations of electrophoretic mobility were made with the same type of rectangular-section micro-cell (10 mm. wide × 0·2 mm. deep) and technique as previously described (Douglas, 1955). All the electrophoretic measurements relate to 25°. All the mobility values reported are mean values (averaged for at least 24 observations in any one determination) except in Fig. 2, where the histograms record the frequency of individual spore velocities.

In making the twenty-four or more observations, one 'stationary level' in the cell (Douglas, 1947) was focused and two spores were timed each way; the other 'stationary level' was then focused and four more timings were made; the first level was refocused, four timings were made—and so on. This gave a minimum of three sets of four observations for each level, located three times; A slight drift velocity, up to 5% of the spore velocity, was sometimes observed in the absence of an applied field; this was allowed for by averaging the reciprocals of the times in each set of four, as standard procedure. The agreement between the sets of observations at the two 'stationary levels' gave a check on cell performance.

RESULTS

Mobility–pH relations for untreated spores. Mobility–pH relations, in Michaelis buffers pH 2–9 and $I = 0{\cdot}05$, for resting spores of the strains of *B. megaterium* and *B. subtilis* used have already been reported (Douglas, 1955). Fig. 1 records the behaviour of further samples of these spores, as determined in the present work, together with the curve for resting spores of *B. cereus* used by Powell and co-workers. The mean mobility values recorded were reproducible to within 2% experimentally.

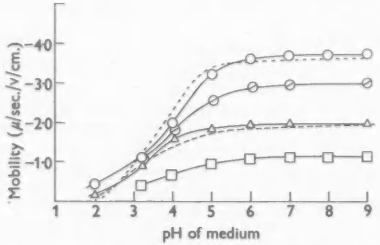

Fig. 1. Mobility–pH curves for resting spores and bacterial peptides in Michaelis buffers, $I = 0{\cdot}05$ at 25°. □, Resting spores of *B. cereus*; △, resting spores of *B. subtilis*; ○, ⊘, different samples of resting spores of *B. megaterium*; – – –, exudate hexosamine peptide adsorbed on Nujol droplets; - - - -, DL-polyglutamic acid (*B. subtilis*) adsorbed on Nujol droplets.

The mobility against pH behaviour was remarkably consistent for samples of *B. subtilis* spores reaped from different cultures; the smaller number of *B. cereus* samples studied showed similar consistency. For *B. megaterium* the different samples studied gave mobility against pH curves of the same form, but varying in quantitative values between the two extreme curves given, which relate to spores reaped from cultures grown at different times. Nevertheless, the several *B. megaterium* samples showed the same response (within 2%) to enzyme treatments, etc., in terms of the relative changes in electrophoretic mobility thus produced.

Fig. 1 also includes the corresponding curves for a hexosamine peptide associated with the spore coat (Strange & Dark, 1956) and liberated into the medium on germination (Powell & Strange, 1953), and a DL-polyglutamic acid, produced extracellularly by vegetative *B. subtilis* (Thorne, 1956). These curves were obtained for the compounds adsorbed from solution in the Michaelis buffers on to liquid paraffin (Nujol) droplets, under conditions giving a complete surface layer (Douglas & Shaw, 1957).

Electrophoretic homogeneity of spore samples. In order to assess the significance of any shift in spore mobilities, both individual and mean, information concerning the electrophoretic composition of the spore suspensions is required, for resting spores and for spores variously treated. In particular the coefficient of variance (V) of individual velocities and the standard error of their mean are required, if possible.

Fig. 2 presents summations of the individual observations from several separate experiments made in Michaelis buffer, pH 7, $I = 0{\cdot}05$, 25°, both for resting spores and treated spores. These histograms are typical representatives of many more relating to other pH values and other spore treatments. The dotted curves are the Gaussian or normal distribution curves fitted to the observations in the usual way.

Electrophoretic mobilities of treated spores. Fig. 3 records the limiting mobility values, in Michaelis buffer, pH 7, at 25°, for ungerminated spores after the different enzyme and control treatments. They are expressed relative to the mobility of untreated resting spores as unity; as will be seen later, this enables the significance of the mobility shifts to

be assessed on sight, from knowledge of the standard error limits for these mean mobility values. For the three organisms, the effects of incubation at 37° with inactivated enzymes were the same as with incubation alone (*I* in Fig. 3). The adsorption experiments at room temperature gave the same final mobilities as the untreated control (*C* in Fig. 3) in all cases. Mobility values at pH 3 were also obtained, to allow for the possibility of coincident behaviour for treated and untreated spores at pH 7 or near, even when some significant change in surface had been produced (Harris, 1953). It was found that when a significant shift in the mobility occurred at pH 7, one was observed at pH 3 also; when none occurred at pH 7 there was none at the lower pH. Accordingly, Fig. 3 records only the findings at pH 7.

DISCUSSION

Before considering the other results presented in this paper it is pertinent to discuss the electrophoretic homogeneity of the initial resting spores, and any changes produced by subsequent treatment. The histograms were always unimodal, as shown in Fig. 2, and corresponded closely to normal distribution curves with a coefficient of variation (*V*) of about 6%. For resting spores of *B. cereus*, as illustrated, the larger variance found (*V* about 9%) may be the result of combining observations on spores of different ages. Newly reaped spores and 1-year-old spores gave mobilities of $-1{\cdot}2$ and $-0{\cdot}9\,\mu$/sec./v/cm. respectively at pH 7. This mobility variation may in turn be associated with the falling off with age of a lytic activity exhibited by these *B. cereus* spores (Strange & Dark, 1957). The other histograms related to spores of similar ages; *B. subtilis* spores showed no variation with age.

As already mentioned, the overall variance (V^2) includes (i) the effect of the Brownian movement, (ii) experimental errors in timing and in locating the 'stationary levels', (iii) any electrophoretic heterogeneity of the spore samples, and possibly (iv) a small error due to imperfect elimination of drift. Even if (i) and (ii) were negligible the homogeneity of the spore samples with respect to electrophoresis, and in turn to their surface composition,

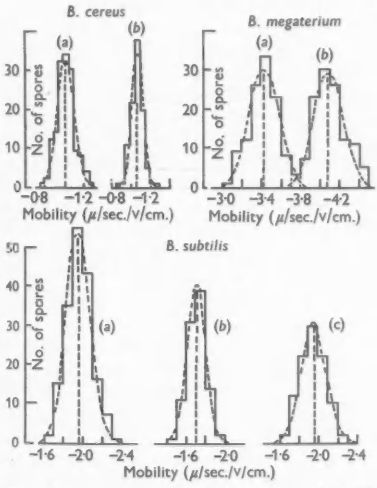

Fig. 2. Histograms of number of spores against electrophoretic mobility for normal and treated spores, and the corresponding Gaussian distributions. Mobilities were determined in Michaelis buffer, pH 7, $I = 0{\cdot}05$ at 25°. *B. cereus*: (*a*) untreated, resting spores; S.D. 0·09; (*b*) trypsin-treated, ungerminated spores; S.D. 0·06. *B. megaterium*: (*a*) untreated, resting spores; S.D. 0·16; (*b*) incubation control, ungerminated; S.D. 0·18. *B. subtilis*: (*a*) untreated, resting spores; S.D. 0·13; (*b*) lysozyme-treated ungerminated spores; S.D. 0·10; (*c*) ribonuclease-treated, ungerminated spores; S.D. 0·13.

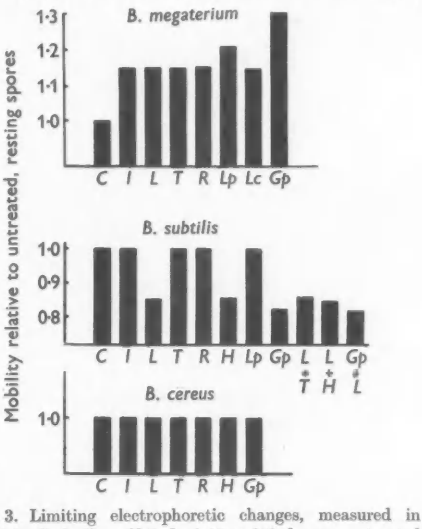
B. megaterium
B. subtilis
B. cereus
Mobility relative to untreated, resting spores
C I L T R Lp Lc Gp
C I L T R H Lp Gp L L Gp
T H L
C I L T R H Gp

Fig. 3. Limiting electrophoretic changes, measured in Michaelis buffer, pH 7, $I = 0{\cdot}05$ at 25°, for spores treated with enzymes in 0·1 M-phosphate buffer, at 37°. Values are relative to those for untreated resting spores. *C*, Untreated, resting spores; *I*, incubation alone; *L*, incubation with lysozyme; *T*, incubation with trypsin; *R*, incubation with ribonuclease; *Gp*, incubation with polyglutamylpeptidase preparation; *H*, incubation with hyaluronidase preparation; *Lp*, incubation with lipase preparation; *Lc*, incubation with lecithinase preparation; *L* + *T*, incubation with lysozyme, followed by incubation with trypsin; *L* + *H*, incubation with lysozyme, followed by incubation with hyaluronidase; *Gp* + *L*, incubation with glutamylpeptidase, followed by lysozyme.

could be considered satisfactory; when allowance is made for (i) and (ii) the degree of uniformity appears very great. In the electrophoretic measurements, the potential gradient was adjusted so that the spores took approximately 8–10 sec. to cover 60 μ at the stationary level. Thus assuming the spores to approximate to spheres of diameter μ and that their diffusion coefficient $D = kT/6\pi\eta r$, the Brownian displacement $\Delta = \sqrt{2Dt}$ is about 3 μ, i.e. 5 % of 60 μ. Hence error (i) contributes about 0·0025 to V^2; this is probably an overestimate as the spores are ellipsoidal and charged. The excursion time can be judged to 0·1 sec. and the stationary level located to better than 1 μ in 40 μ (cell depth 200–250 μ); thus error (ii) may contribute about 0·0005 to V^2. The observed V^2 is nearly always about 0·0036; deducting contributions from errors (i) and (ii), and remembering that there may still be a small residual drift error (iv), we conclude that the true coefficient of variation of the mobility is not more than 2·5 %. In fact, since Sheppard's correction for grouping has not been applied in the above general argument, it is possible that the spores are strictly homogeneous with respect to electrophoretic mobility. It is of interest to note that hydrocarbon droplets also show a coefficient of variation of about 6 % in Michaelis buffer, pH 7 and $I = 0{\cdot}05$, the same apparatus and technique being used. Further, the remarkable homogeneity suggested for the resting spores appears to be maintained after the various treatments to which the spores have been subjected in the present work.

Although no impressively large changes in mobility result from enzyme treatment (Fig. 3) the shifts of 15 % or more observed for some enzymes are significant. The standard error of the mean mobilities ($\sim V/\sqrt{n}$, where n is the number of individual observations in a mobility determination) reported here is about 1·5 % at a maximum. Electrophoretic shifts were observed only with spores of *B. megaterium* and of *B. subtilis*. For *B. subtilis* spores lysozyme, hyaluronidase and the glutamylpeptidase separately produced significant shifts; the combinations of enzyme treatments were used in an attempt to enhance these shifts, but without any very marked effect. *B. megaterium* spores showed a pronounced shift with the glutamylpeptidase, a lesser shift with the lipase preparation, but none with lysozyme, etc. *B. cereus* was unco-operative throughout.

Since the electrophoretic behaviour of a particle surface reflects its chemical nature, it is reasonable to assume that the electrophoretic shifts reflect changes in the spore surfaces produced by enzyme attack. It is known that both lysozyme and hyaluronidase attack polysaccharide substrates rich in *N*-acetylhexosamine, whereas the glutamylpeptidase is reasonably specific for polypeptides containing glutamic acid (Thorne, 1956). It is therefore suggested that the surface of *B. subtilis* spores includes both *N*-acetylhexosamine-containing and glutamylpeptide-containing material and that the *B. megaterium* spore surface contains a glutamylpeptide component and possibly some lipid.

This view is supported by the mobility–pH curves for model particles having adsorbed surface layers of a hexosamine peptide or DL-polyglutamic acid, which closely resemble the curves for *B. subtilis* and *B. megaterium* spores respectively (Fig. 1). From the known surface and interfacial-film behaviour of polysaccharides and polypeptides (Davies, 1953), it seems probable that when adsorbed on oil droplets the hexosamine peptide will be orientated with both components in the surface and with any charged or polar side-groups preferentially in the aqueous medium; polyglutamic acid is likely to behave similarly, especially when the carboxyl groups can ionise.

It is thus possible that the outermost surface layers of the resting spores of both *B. subtilis* and *B. megaterium* consist essentially of this hexosamine peptide, which is largely liberated by these organisms on germination, orientated differently so that both its hexosamine and peptide parts appear in the surface of *B. subtilis*, but only the peptide part appears in that of *B. megaterium*.

These suggestions are consistent also with the action of enzymes on the isolated spore coats of these organisms and on the exudate peptide. Thus Strange & Dark (1956) have shown that lysozyme accelerates the liberation of the hexosamine peptide from the spore coats and also leads to degradation of the hexosamine peptide itself, as indicated by diffusion through a cellophan sac. Further, the glutamylpeptidase used in the present work was subsequently found to degrade the hexosamine peptide with the production of smaller peptides (Strange & Thorne, 1957).

Despite the negative response to enzymes, the mobility against pH for *B. cereus* spores is similar in form to that for the other spores and suggests that they have the same surface-charge groups, $-CO_2H$, at lower density. Essentially the same hexosamine peptide is associated with the spore wall and liberated on germination (Strange & Dark, 1956). The surface of *B. cereus* spores might also contain hexosamine peptide, orientated in a way different from those suggested for *B. subtilis* and *B. megaterium*, so that the enzyme-sensitive links in the hexosamine peptide are shielded from external attack. Alternatively, the exosporium may be the cause of the lack of response with *B. cereus*. This is rather speculative, but a recent study by the authors (Douglas & Parker, 1957) of the cation-charge-reversal spectra of the resting

spores of the three organisms, and of the hexosamine peptide adsorbed on oil droplets, again suggests closely similar chemical compositions for their surfaces, with $-CO_2H$ predominating as the effective charge group (Kruyt, 1949).

Finally, the relative resistance of the spores to the variety of enzymes used stands in marked contrast to the sensitivity of the vegetative cells to at least some of these enzymes. Thus *B. megaterium* spores show no electrophoretic shift on treatment with lysozyme; further, they appear unchanged by phase-contrast microscopy and remained ungerminated and viable. On the other hand, vegetative cells of *B. megaterium* are well known for their sensitivity to lysozyme (Welshimer, 1953; Wiebull, 1953; Salton, 1955). This has been confirmed for vegetative cells grown from the present spores (Douglas & Parker, 1958). The insensitivity of the resting spores to enzymes is paralleled by their relative resistance to a variety of bactericidal agents, and is perhaps to be expected if the function of spore formation is regarded as protective (Williams, 1952). The problem remains of explaining this insensitivity in terms of the chemical and physical composition of the spore coat, which appears to be somewhat similar to that of the vegetative cell wall (Salton, 1955).

SUMMARY

1. Change in electrophoretic behaviour has been used to assess the susceptibility of the surfaces of resting spores of *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus cereus* to attack by a variety of enzymes including lysozyme, trypsin and lipase.

2. *B. subtilis* spores were affected by lysozyme, hyaluronidase and a polyglutamylpeptidase, *B. megaterium* by the glutamylpeptidase and lipase, *B. cereus* by none of the enzymes.

3. The results, taken in conjunction with the mobility–pH curves for the resting spores and for bacterial peptides, are consistent with the surfaces of the resting spores being composed essentially of differently orientated layers of the same hexosamine peptide, which is partially liberated from the sporecoats on germination.

4. Analysis of histograms of mobilities against numbers of spores shows the initial spore samples to be remarkably homogeneous electrophoretically, and that this homogeneity is maintained after the various enzyme treatments.

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Electrophoretic Studies on Bacteria

3. THE GROWTH CYCLE OF *BACILLUS MEGATERIUM*, THE BEHAVIOUR OF CELLS AND THE CHANGES PRODUCED BY LYSOZYME*

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In previous studies some attempt was made to characterize and determine the composition of the outermost surface layers of resting spores of *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus cereus* electrophoretically, by measuring their mobility–pH behaviour in buffered media (Douglas, 1955) and the changes produced by treatment with a variety of enzymes (Douglas & Parker, 1958). In independent biochemical studies, a hexosamine-containing peptide of characteristic composition was shown to be a common constituent of spore coats of these species (Strange & Dark, 1956). It

* Part 2: Douglas & Parker (1958).

was concluded that, in all three species, the observed electrophoretic behaviour was consistent with the spore surface being mainly composed of this hexosamine peptide and that the differences in behaviour between spores of the three species could be accounted for by different orientations or complex-formation of the peptide in their surface layers.

In general, resting spores were remarkably resistant to enzyme attack in contrast to the behaviour of vegetative organisms. In particular, resting spores of *B. megaterium* were completely resistant to lysozyme, in striking contrast to the rapid attack and lysis reported for vegetative cells of the same species under similar conditions (Weibull, 1953; Salton, 1953). This indicated marked differences in surface composition between spores and vegetative cells and suggested corresponding differences in electrophoretic behaviour. Accordingly, a study has been made of the changes in electrophoretic behaviour and susceptibility to lysozyme occurring during the complete growth cycle of *B. megaterium* in two media of very different constitution. Parallel pH, electrical-conductivity and optical-density measurements were also made.

EXPERIMENTAL

The spores used to inoculate the culture media were of the same laboratory strain of *B. megaterium* as those used by Powell and co-workers in a series of biochemical studies (e.g. Spencer & Powell, 1952; Powell & Strange, 1953; Strange & Powell, 1954). They were kindly supplied by Mrs J. F. Powell as a suspension of well-washed spores in sterile water (2×10^{10} spores/ml.).

The first medium in which the growth cycle was studied was a sterilized meat extract–peptone medium (Tarr, 1933) containing (%, w/v): peptone 1 (Parke, Davis and Co. Ltd.), Lab-Lemco 0·2, NaCl 0·3 and glucose 0·2; the pH was adjusted to 7·3 with NaOH. The medium was freshly prepared for each experiment. The second medium used was a sterilized lactate + glucose + salts solution of the following composition (%, w/v): glucose 0·25, ammonium lactate 0·125, ammonium phosphate 0·07, NaCl 0·05, KCl 0·05, $MgSO_4$ 0·1, ferric ammonium citrate 0·0017 and $MnSO_4$ 0·0003; all percentages are based on anhydrous salts. The calculated ionic strength (I) of both media, on the basis of this salt content, was approx. 0·05.

In order to follow the electrophoretic behaviour of the developing organisms continuously, experimental arrangements were made to circulate the growing culture from an upper culture vessel through the electrophoresis cell, or a by-pass, to a similar lower culture vessel and back to the upper vessel. Polythene tubing was used to join the various parts. Each of the culture vessels consisted of a 1 l. separating funnel stoppered by a rubber bung through which passed a sampling siphon, a thermometer, an air-escape tube with a sterile cotton-wool plug and an aeration tube. The air supplied to the culture vessels, from the compressed-air supply, first passed through a sterile cotton-wool filter and was presaturated with water vapour in order to minimize volume changes during an experiment, which sometimes continued for 4 days. The whole arrangement was set up inside a working cabinet, some 6 ft. cubed, which also housed the operator. In order to maintain the culture medium, the electrophoresis cell, etc., at 37° it was necessary to regulate the air temperature in the cabinet to about 40°. The design and operation of the micro-apparatus has been previously described (Douglas, 1955). Phase-contrast optics was used for all observations, with a ×40 objective and a ×15 micrometer eyepiece.

For the electrophoretic studies it was desirable that at any time the culture should contain as high a proportion as possible of organisms at the same stage of development. It was found that inoculation of the main culture volume with young vegetative cells, produced in a separate small volume of the same medium, resulted in much more homogeneous cultures than those obtained by direct inoculation with spores, and the following procedure was therefore adopted.

The spores from 1 ml. of the stock suspension (2×10^{10} spores) were washed twice with sterile water, by centrifuging, and resuspended in 1 ml. This inoculum was then added to 250 ml. of the appropriate medium (Tarr or lactate) in a third 500 ml. incubation vessel, similar to those previously described, and maintained at 37°, and air supplied at a rate sufficient to provide vigorous agitation. Measurements of the initial electrophoretic mobility of spores and early vegetative forms in the medium were made by coupling this initial small-scale culture vessel to the electrophoresis cell and running small volumes through, which were then used for optical-density, electrical-conductivity and pH measurements. These were made at 25° with respectively an EEL absorptiometer (Evans Electroselenium Ltd.) at 600 mμ, a Mullard conductivity cell with a modified Shedlovsky bridge and a glass electrode in conjunction with a valve potentiometer.

After a few hours 100 ml. of the remaining culture volume was siphoned off, and the young cells were centrifuged and then washed with and resuspended in 0·05 M-NaCl (20 ml.). While the young vegetative cells were being treated, the electrophoresis cell was flushed through with fresh culture medium and coupled to the main culture system for the rest of the culture period. Then 10 ml. of cell suspension was added to 750 ml. of the aerated culture medium at 37° in each of the two main culture vessels. This gave a sufficient number of young cells/ml. to make electrophoretic measurements from the time of inoculation onwards. The medium in the two culture vessels was continuously and vigorously aerated, and passed between them every 30 min. (flowing from the upper to the lower vessel through the by-pass, or to the electrophoresis cell for measurement, under gravity and being returned by the pressure of the aeration supply to the lower vessel when its escape valve was closed). Periodically 50 ml. samples were siphoned off for the determination of optical density, pH and electrical conductivity.

In determining the mobility–pH of cells from cultures of various ages, a volume of culture containing approx. 10^{11} cells (as estimated from the optical density) was siphoned off from a culture of the appropriate age, the cells were centrifuged, washed twice with 0·05 M-NaCl to remove any culture components and resuspended in 10 ml. of this solution. This stock cell suspension was used to prepare a

series of dilute suspensions (1 ml. of stock/100 ml.) in Michaelis buffers, $I = 0{\cdot}05$, covering the pH range 2·5 to 9. The electrophoretic mobilities of cells in these dilute suspensions were then determined at 25°, a second micro-electrophoresis apparatus housed in another cabinet being used, in the manner described in earlier papers relating to resting spores (Douglas, 1955; Douglas & Parker, 1958).

The effect of lysozyme on washed cells from cultures of different ages was observed at 25° both electrophoretically and by measurement of changes of optical density. A portion was siphoned off from a culture of the appropriate age, centrifuged, washed twice with and finally resuspended in a small volume of 0·05 M-NaCl. Preliminary optical-density measurements and phase-contrast observation (at ×1500) showed that, at 25° and pH 7, lysozyme attacked cells with equal facility in phosphate or Michaelis buffer, and that reasonably extended observation of the changes produced was possible for final enzyme concentrations between 5 and 50 μg./ml.

For the quantitative optical-density measurements a portion of the stock cell suspension in 0·05 M-NaCl was centrifuged and the cells were resuspended in either phosphate or Michaelis buffer, pH 7, $I = 0{\cdot}05$ (Douglas, 1955), to give a preliminary optical density in a 1 cm. cell of approx. 0·5, relative to buffer alone. A volume (9 ml.) of this buffered suspension was then placed in each of two 1 cm. cells, and 1 ml. of buffer only added to one cell and the optical-density reading for this control suspension reset to unity; 1 ml. of an appropriate solution of lysozyme in the same buffer was then added to the other 9 ml. of suspension and the optical-density readings for both cells were followed with time.

For the electrophoretic measurements the washed cells in 0·05 M-NaCl were centrifuged and resuspended in Michaelis buffer, pH 7, $I = 0{\cdot}05$, to give approx. 10^8 cells/ml. From 100 ml. of this suspension, 5 ml. was removed and replaced by 5 ml. of the same buffer containing lysozyme; after mixing, this suspension was introduced into the micro-electrophoresis cell, previously filled with buffer only, and the mobility of the *B. megaterium* cells determined at a number of time intervals.

It was found, in agreement with other workers (Weibull, 1953), that lysozyme removed the cell wall less rapidly with increasing age of the culture from which the cells were taken. The resulting protoplasts were unstable in the buffer solution alone. Experiments were therefore repeated in buffers containing M-sucrose, in which the protoplasts were quite stable. After incubation with enzyme the resulting protoplasts were centrifuged, washed with and resuspended in Michaelis buffer, $I = 0{\cdot}05$, containing M-sucrose, and their mobility–pH behaviour was determined in the micro-cell at 25°.

Finally, samples of the spores obtained at the end of the culture cycle were centrifuged, washed repeatedly with 0·05 M-NaCl, resuspended in Michaelis buffer, $I = 0{\cdot}05$, and their mobility–pH was determined at 25°.

The buffer solutions used were made up in water with A.R. salts and HCl, and were sterilized before being used. The lysozyme was crystallized material supplied by Armour Chemicals Ltd., Hampden Park, Eastbourne, Sussex.

RESULTS

The main results are summarized in Figs. 1–7, the legends to which give details. In addition to the measurements recorded in Figs. 1 and 2, the electrical conductivities of the media at 25° were also determined. They showed little variation (about 5 %) throughout the culture periods for both media. These conductivity values were used to calculate the electrophoretic mobilities given in Figs. 1 and 2, which were thereby corrected to 25°.

In general, parallel changes were observed in the optical density, electrical mobility and its variation with pH in the two media at corresponding stages during growth and sporulation, which proceeded approximately twice as fast in the lactate medium

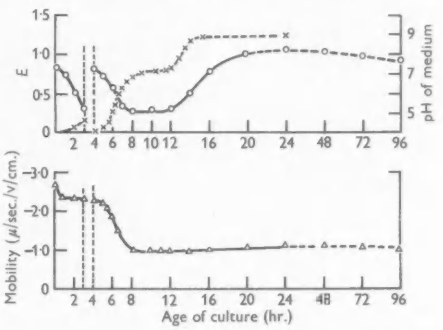

Fig. 1. Growth of *B. megaterium* in Tarr medium at 37°. ×, Optical density (600 mμ) against age of culture, measured at 25°; ○, pH of medium against age of culture, measured at 25°; △, electrophoretic mobility against age of culture, measured in medium on 6–12 μ rods, corrected to 25°. The vertical dotted lines indicate the transfer period mentioned in the text.

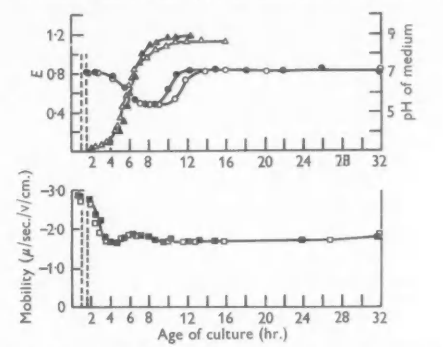

Fig. 2. Growth of *B. megaterium* in lactate medium at 37°. △, ▲, Optical density (600 mμ) against age of culture for two typical cultures, measured at 25°; ○, ●, pH of medium measured at 25°, for same two cultures; □, ■, electrophoretic mobility against age of culture, measured in medium on 6–12 μ rods, corrected to 25°. The vertical dotted lines indicate the transfer period.

as in the Tarr medium. The changes in electrophoretic mobility and optical density are correlated with those in phase-contrast appearance. On germination there was a small fall in mobility, which then remained constant as vegetative cells developed and slowly multiplied during the lag phase. When growth became rapid, and long vegetative chains appeared (up to 60 μ and more long in Tarr medium), the electrophoretic mobility began to fall steeply and independently of the chain length. After a few hours (compare Fig. 1), with the general development of dividing walls, the chains separated into smaller parts of which the majority were 6–15 μ in length. For a period during the phase of rapid growth the organisms became highly motile and electrophoretic measurement became difficult or impossible. Within an hour, however, this motility died away and the electrophoretic mobility ceased to fall as the organisms began to show internal granular detail. In both media the electrophoretic mobility remained approximately constant from this stage onwards as spores developed, and finally free spores predominated in the culture. This happened after about 24 hr. growth in the lactate medium and after 48 hr. in the Tarr medium. In the latter, the experiment was continued to determine whether the mobility of the spores would in time rise to the initial spore value. It remained low, however, and, as is recorded later, the initial spore value was attained only after repeated washing.

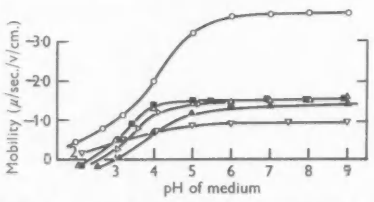

Fig. 3. Mobility–pH curves for *B. megaterium* cells from cultures of different ages, measured in Michaelis buffers, $I = 0{\cdot}05$ at 25°. ▲, Washed cells from 12 hr. Tarr medium; ▽, formalin-treated cells from 12 hr. Tarr medium; ▷, washed cells from 18 hr. Tarr medium; △, washed cells from 30 hr. Tarr medium; ■, washed cells from 12 hr. lactate medium; ○, resting *B. megaterium* spores (Porton sample).

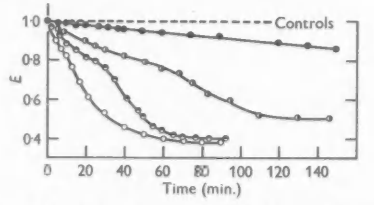

Fig. 4. Effect of lysozyme on *B. megaterium* cells from Tarr cultures. Final lysozyme concn.: 5 μg./ml. Phosphate or Michaelis buffer or both, pH 7, $I = 0{\cdot}05$. Temperature 25°. ○, Cells (3¼ hr.) from Tarr medium; ◓, cells (6 hr.) from Tarr medium; ◑, cells (12 hr.) from Tarr medium; ●, cells (30 hr.) from Tarr medium; – – –, controls in the absence of enzyme.

Fig. 3 records the mobility–pH of washed cells from both Tarr- and lactate-medium cultures of different ages together with the mobility–pH curve for the initial resting spores. There was some variation in the mobility of the vegetative cells as the age of the culture increased but cells from both media gave the same limiting relationship of mobility and pH. The cells were studied after washing with 0·05 M-NaCl alone, since, with cells from a 12 hr. culture in Tarr medium for example, it was found that 'fixing' with 1 % formalin led to a marked change in mobility against pH (Fig. 3). Washed cells killed by heating at 100° for 15 min. showed essentially the same behaviour as that recorded for washed unheated cells.

Fig. 4 shows the variation in optical density with time of suspensions of washed cells, from Tarr cultures of different ages, treated with 5 μg. of lysozyme/ml. at 25°, and the constancy of the optical density in the absence of enzyme. Allowing for an increase in the final optical density with the internal development of cells in older cultures, the rate of attack fell off with increasing age of the culture. This was confirmed by phase-contrast observation. With time, the cell wall appeared to swell gradually and become gel-like until the protoplasm lost its original outline and became spherical, still within the gel layer. Subsequently, with the secondary fall in optical density recorded in Fig. 4, some of the protoplasts were observed to disintegrate.

Fig. 5 records the effect of lysozyme at different concentrations (5, 10 and 50 μg./ml.) on washed cells of *B. megaterium*, at pH 7 and 25°, both in the absence and presence of M-sucrose. The initial rates of decrease of optical density remained unchanged in the presence of M-sucrose, but the secondary fall off was eliminated, as is clearly shown for cells from a 12 hr. Tarr culture.

No significant change in electrophoretic mobility of the organisms with time was observed in the presence of lysozyme until, in the absence of sucrose, protoplasts began to burst. In the presence of sucrose, the mobility remained sensibly unchanged at the original cell value so long as the gel layer remained undisturbed. On centrifuging and washing in buffer + M-sucrose, however, a suspension of separate protoplasts apparently free of the gel layer was obtained, and the mobility–pH of these was markedly different from that of the

original cells. Fig. 6 presents these results for protoplasts, the mobilities both as observed and as recalculated for a medium of the same viscosity as water being recorded; for comparison, the limiting curve for untreated cells in the absence of sucrose is also recorded.

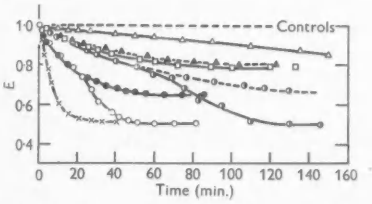

Fig. 5. Effect of lysozyme at different concentrations on *B. megaterium* cells, with and without M-sucrose. Phosphate or Michaelis buffer or both, pH 7, $I = 0{\cdot}05$. Temperature 25°. ◑—◑, Lysozyme (5 μg./ml.) on 12 hr. cells (Tarr medium); ◑---◑, lysozyme (5 μg./ml.) on 12 hr. cells (Tarr medium) + M-sucrose; ○, lysozyme (10 μg./ml.) on 12 hr. cells (Tarr medium); ●, lysozyme (10 μg./ml.) on 12 hr. cells (Tarr medium) + M-sucrose; ×, lysozyme (50 μg./ml.) on 12 hr. cells (Tarr medium); △, lysozyme (5 μg./ml.) on 30 hr. cells (Tarr medium); ▲, lysozyme (10 μg./ml.) on 30 hr. cells (Tarr medium); – – –, lysozyme (10 μg./ml.) on 30 hr. cells (Tarr medium) + M-sucrose; □, lysozyme (10 μg./ml.) on 12 hr. cells (lactate medium).

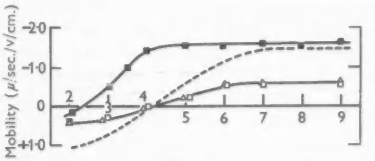

Fig. 6. Mobility–pH curves for protoplasts, in Michaelis buffers + M-sucrose, at 25°. △, Protoplasts from 30 hr. cells (Tarr medium); □, protoplasts from 12 hr. cells (lactate medium); – – –, curve corrected to viscosity for water; ■, 12 hr. cells from lactate medium.

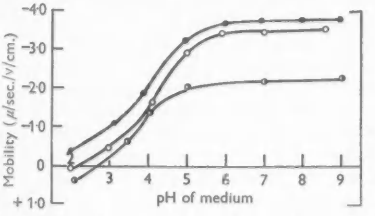

Fig. 7. Mobility–pH curves for *B. megaterium* spores. Michaelis buffer, $I = 0{\cdot}05$. Temperature 25°. ○, Resting spores, typical sample; ◑, spores from lactate culture, washed four times in NaCl soln.; ●, spores from lactate culture, washed eight times in NaCl soln.

Finally, in Fig. 7 the variation of mobility with pH of the spores obtained in the cultures is compared with that of the initial spores. Only after the spores had been repeatedly spun down and resuspended in 0·05N-NaCl did their behaviour approximate to that of the initial spore sample, which is included in the figure.

DISCUSSION

From these results it is clear that, allowing for the time factor, the development of *B. megaterium* followed the same course in the Tarr and in the lactate medium, producing cells of the same surface properties and eventually spores which, when washed, closely resembled the initial resting spores.

Figs. 1 and 2 record marked variations in the electrophoretic behaviour of the developing organisms during the first half period of the growth cycle and it may be assumed, in accordance with evidence from model surface behaviour (e.g. Douglas & Shaw, 1957), that this reflects changes in the composition, ionic in particular, of their outermost surface layers. The initial drop in mobility on germination was small and may perhaps reflect little more than an increase of volume and a slight expansion of surface substance the same as that of resting spores. The results of a detailed study of germinated spores support this suggestion.

As vegetative cells proliferated, however, during the period of rapid growth there was a striking decrease in electrophoretic mobility to a lower value which remained constant when rapid growth ceased. This suggests that considerable changes in the surface composition took place at this time, after which the cell surface remained effectively constant in composition although, as indicated by the enzyme results, the cell wall increased in thickness. The mobility–pH curves for resting spores and for washed cells given in Fig. 3, together with results in Figs. 4 and 5, support these suggestions. The constancy of the mobilities over the pH range 5–9 appears to preclude the possibility that the observed decrease of mobility might have been due to the concomitant fall in pH to about pH 5·5 and its recovery again later, which was observed at this stage in the growth cycle. The ionic strengths of the two media were approximately equal, constant and the same as for the Michaelis buffers in which the washed cells were studied. The mobility of cells in the lactate medium agreed quite well with the value for washed cells in Michaelis buffer of about the same pH; the mobility in Tarr medium, however, was considerably lower and the authors suggest that this was due to adsorption of some component from this complex medium, which was desorbed on repeated washing with 0·05N-NaCl.

Additional evidence of considerable differences, biological if not chemical, between the effective surfaces of well-washed resting spores and vegetative cells of *B. megaterium* is afforded by their response to lysozyme. Whereas resting spores of *B. megaterium* were unaffected by incubation with 50 μg. of lysozyme/ml. for up to 24 hr. at 37° (Douglas & Parker, 1958), vegetative cells of various ages were attacked by even 5 μg. of lysozyme/ml. at 25° within much shorter times. This suggests differences in the nature or orientation of the molecular material in the surface, in addition to the differences in the number, and perhaps nature, of the effective charge groups, between spores and vegetative cells of this organism.

Evidence of the possible chemical nature of the surface substance of *B. megaterium* vegetative cells is provided by the mobility–pH curve, which suggests an acid polysaccharide with free–CO_2H groups (Douglas & Shaw, 1957); it bears a considerable resemblance to the curves for resting spores of *B. subtilis* and for the hexosamine peptide liberated by spores of both *B. megaterium* and *B. subtilis* during germination (Powell & Strange, 1953; Strange & Powell, 1954). Further, the cation–charge-reversal spectra (Bungenberg de Jong, 1949) of *B. megaterium* spores and vegetative cells and of a number of 'carboxyl' substances (polysaccharide, peptide and other types of molecule) adsorbed on hydrocarbon droplets, have been determined (Douglas & Parker, 1957). These have confirmed the presence of $-CO_2H$ as the principal surface-charge group for both *B. megaterium* vegetative cells and spores, but have not given an equally clear indication of the nature of the molecule with which it is associated. In this connexion it is of interest to note that the spectrum obtained for vegetative cells of *B. megaterium* showed, for ions common to both, considerable similarities to that reported for *Escherichia coli* (Davies, Haydon & Rideal, 1956), from which the authors concluded that the surface of *E. coli* was composed of an acid polysaccharide, possibly arabic acid or an arabinate.

Bearing in mind the results reported by Salton (1953, 1955) for the composition of the isolated cell walls of another strain of *B. megaterium*, for which glucose, hexosamine, a limited number of amino acids including glutamic acid, and diaminopimelic acid were constituents identified in cell-wall hydrolysates, and the results presented above, it may be concluded that the outermost surface layers of cells of the strain of *B. megaterium* studied here are probably composed of a mucopolysaccharide, possibly a hexosamine peptide similar to but not identical with that exuded from spores of the same organism (Strange & Powell, 1954).

Mitchell & Moyle (1956) have shown that in *Staphylococcus aureus* the protoplast membrane is composed of lipoprotein. The mobility–pH behaviour of protoplasts of *B. megaterium* recorded in Fig. 6 is consistent with their surface also being effectively lipoprotein or protein (Douglas & Shaw, 1957). Treatment with trypsin (Armour crystallized) resulted in a spectacular disintegration of the protoplasts, leaving granules in suspension when these had been present in the original cells.

Finally, the observation that the mobility–pH of the spores produced at the end of the growth cycle was the same as that of vegetative cells in the culture media suggests the persistence of cell-wall substance round the spores even after the apparent disintegration of the sporangia. It cannot be decided from the results whether or not this adsorbed material, which was not visible under phase contrast at $\times 1500$, could be completely removed by repeated washing, but if some finally remains it must be regarded as a normal part of the spore coat, and one which may contribute to the spore behaviour both electrophoretically and biologically. There was, however, no evidence of a definite outer structure or exosporium analogous to that which appears to be an integral part of spores of *B. cereus* (Robinow, 1951).

SUMMARY

1. A study has been made of the changes in electrophoretic behaviour and susceptibility to lysozyme occurring during the growth cycle of *Bacillus megaterium* in meat extract–peptone and in lactate–glucose media. Parallel pH, electrical-conductivity and optical-density measurements have also been made. Samples of vegetative cells have been withdrawn from cultures of different ages and the variation of their mobility with pH determined together with the effect of lysozyme, which led to the production of protoplasts.

2. The same pattern of electrophoretic change was observed during growth in both media. Little change in electrophoretic mobility occurred until the phase of rapid vegetative growth, when the mobility fell to a much lower value and thereafter remained constant up to and including the formation of spores, free of sporangia. On repeated washing the spore behaviour approximated to that of the original spores. Lysozyme sensitivity was greatest for vegetative cells from young cultures and decreased with increasing age of the cultures.

3. It was concluded that marked changes in surface composition occurred during the phase of rapid vegetative growth and that, whereas the composition of the cell walls remained constant thereafter, their thickness increased with age.

From the variation of mobility with pH, the principal surface-charge group of vegetative cells appeared to be carboxyl, possibly associated with a hexosamine-peptide substance. The behaviour of the protoplasts was consistent with their having a lipoprotein or protein surface.

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Studies on the Amide and *C*-Terminal Residues in Proteins

1. THE CHARACTERIZATION OF THE *C*-TERMINAL RESIDUE

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A normal open peptide chain carries an *N*-terminal and a *C*-terminal residue, so that it should be possible to determine the number of such chains in a protein by estimating one or other of these residues. Much of the information at present available has been obtained by applying the dinitrophenyl (DNP) method of Sanger (1945), but with certain proteins this method, like others designed for the same purpose, has so far failed to reveal the presence of any *N*-terminal residue at all, which has led to the suggestion that proteins may contain cyclopeptide chains. The need for complementary procedures dealing quantitatively with the opposite end of the peptide chain is thus apparent, and although carboxypeptidase and hydrazinolysis have indeed been fairly extensively used for the purpose in recent years, both are now known to have their limitations. (For review, see Bailey, 1954).

When the present research was started in 1951 it was realized at the outset that the identification and estimation of the *C*-terminal residues in a protein would not necessarily indicate the full number of peptide chains in the molecule, for in addition to the reservation on cyclic structures mentioned above, the issue might be further complicated by the presence of terminal amide residues. Such a possibility served to emphasize how little experimental evidence had been then adduced in favour of the so-called amide hypothesis, which was first adumbrated by Ritthausen (1872) and came into prominence early in the present century. According to this, the ammonia given on mild hydrolysis of proteins was derived exclusively from asparaginyl and glutaminyl residues in the molecule and, although direct evidence for the presence of these two residues in edestin and gliadin respectively was obtained many years ago, it still remains true that for lack of a suitable method for their quantitative estimation the question of whether an amide such as glycinamide, now known to be a constituent of oxytocin, might be present as a terminal residue has really been left open. With this particular point in view, the aim of the present investigation has been to characterize amide as well as *C*-terminal residues under conditions which would, if possible, be of general application in protein chemistry.

The procedure that has been under investigation is based on the observation of Nystrom, Chaiken & Brown (1949) that under the appropriate conditions lithium borohydride ($LiBH_4$) would reduce the ester group to the corresponding hydroxyl group, but was without action on the acid amide group. It was anticipated that if the free carboxyl groups in a protein could be first esterified and then reduced in this way, the various dicarboxylic acid and *C*-terminal residues originally present would become modified and after hydrolysis of the treated protein would be present in the hydrolysate as

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amino alcohols or hydroxyamino acids (Table 1). Quantitative analysis of this hydrolysate would then provide the data for characterizing the amide and *C*-terminal residues in the protein.

Our preliminary results were encouraging, and the distribution of the amide N in insulin between the asparaginyl, glutaminyl and *C*-terminal asparagine residues was resolved in apparent conformity with the contemporary work of Sanger and his colleagues. Attempts to characterize more completely the *C*-terminal residues, however, led to results which, in the light of the latter workers' findings, were confusing. For clarity in presentation we refrain from quoting in Table 2 the results obtained by the out-dated procedures of these early investigations and give, instead, those of a series of observations by the methods in current use (Rees, 1958). Alaninol, representing the *C*-terminal residue of the insulin *B* chain, was readily identified in full measure (Table 2, no. 3), but ethanolamine and small amounts of both leucinol and tyrosinol were also found (Chibnall & Rees, 1951, 1952). It seemed at the time that these latter products must either represent *C*-terminal residues of protein impurities, which the progress of Sanger's work with the same preparation of insulin was making less and less probable, or must indicate that we were dealing with a side reaction inimical to our purpose.

All the preparations of insulin we have handled, when finely ground and passed through the reduction procedure, gave a β-amino alcohol fraction of negligible magnitude (Table 2, no. 1). As we had already observed an increase in amino N (Van Slyke) on esterifying insulin with methanolic hydrochloric acid, we considered the possibility of an *N–O* acyl shift at serine and threonine residues (Chibnall & Rees, 1951, 1952; Chibnall, Mangan & Rees, 1958c), the *O*-peptidyl esters thus produced undergoing reduction to give the adventitious amino alcohols. Indeed the demonstration by Sanger & Tuppy (1951) of the sequence -glycyl-seryl- in the *B* chain made the hypothesis attractive. To probe more deeply we blocked the hydroxyl groups of the serine and threonine residues by preparing *O*-acetyl insulin. This was esterified with diazomethane, but the reduced ester gave the same array of amino alcohols as before. There remained the possibility that an acyl shift was conditioned by the suppression of the carboxyl groups on reduction (Chibnall & Rees, 1953).

The probable explanation of the enigma was provided by Crawhall & Elliott (1955) who showed that, under the conditions they employed, $LiBH_4$ could bring about a reductive cleavage of peptide bonds in lysozyme to produce amino alcohols. We had ourselves considered the possibility of such a direct action by the reagent, but had rejected it for reasons that seemed decisive at the time. As has already been mentioned, insulin itself appeared to undergo a negligible amount of reduction, and the same was true of β-lactoglobulin. In addition, Bailey (1955) had found that *N*-acetamidoethanol in tetrahydrofuran solution, when treated with $LiBH_4$ for 3 hr. under reflux, gave only 1·5% of *N*-ethylamino alcohol. Likewise we had found that glycylglycyl-α-aminomyristic acid, when submitted to our standard reduction treatment in tetrahydrofuran solution, exhibited peptide cleavage to the extent of only 1·3% of one group. These two substances, unlike the proteins mentioned, were soluble in the reaction mixture, yet in spite

Table 1. *Comparison between the various dicarboxylic acid, dicarboxylic acid amide, and* C*-terminal residues present in the intact protein with the corresponding products present in the hydrolysate of the reduced protein ester*

The following abbreviations have been used: γ-α-HAB, γ-hydroxy-α-aminobutyric acid; γ-β-HAB, γ-hydroxy-β-aminobutyric acid; δ-α-HAV, δ-hydroxy-α-aminovaleric acid; δ-γ-HAV, δ-hydroxy-γ-aminovaleric acid.

Residue in protein	Corresponding product in the hydrolysate of the reduced protein ester
Glutaminyl, *N*-terminal or in chain	Glutamic acid
Isoglutaminyl, *N*-terminal or in chain	Glutamic acid
Glutamine, *C*-terminal	δ-γ-HAV
Isoglutamine, *C*-terminal	δ-α-HAV
α-Glutamyl, *N*-terminal or in chain	δ-α-HAV
γ-Glutamyl, *N*-terminal or in chain	δ-γ-HAV
Glutamic acid, *C*-terminal	Glutamidiol
Asparaginyl, *N*-terminal or in chain	Aspartic acid
Isoasparaginyl, *N*-terminal or in chain	Aspartic acid
Asparagine, *C*-terminal	γ-β-HAB or lactone
Isoasparagine, *C*-terminal	γ-α-HAB or lactone
α-Aspartyl, *N*-terminal or in chain	γ-α-HAB or lactone
β-Aspartyl, *N*-terminal or in chain	γ-β-HAB or lactone
Aspartic acid, *C*-terminal	Aspartidiol
Monoamino or basic amino acid, *C*-terminal	β-Amino alcohol
Monoamino or basic amino acid amide	Monoamino or basic amino acid

of this they had undergone but little change. Nevertheless we prepared, by appropriate solution and precipitation, a sample of insulin which we hoped would be in a finer state of dispersion than we had formerly employed. On reduction this underwent reductive cleavage to a degree that we had not hitherto observed (Table 2, no. 2), confirming the results of Crawhall & Elliott and offering an explanation for the origin of the adventitious amino alcohols mentioned above. This means, in effect, that the changes listed in Table 1 need amplifying, because under conditions of reductive cleavage endopeptide residues will simulate *C*-terminal residues. The outcome, nevertheless, does not vitiate the procedure as we now apply it to proteins of low molecular weight like insulin where the overall interference is relatively small (Tables 2 and 3). With increase in molecular weight, however, the degree of uncertainty in the interpretation of the data becomes proportionately greater and our studies suggest that the profitable limit has already been reached in the multi-chain protein, β-lactoglobulin (Table 5).

As the work progressed and it was found that side reactions were interfering with the full exploitation of the procedure, it became necessary to devote considerable time *inter alia* to the quantitative analysis of certain β-amino alcohols and the four hydroxyamino acids listed in Table 1. β-Amino alcohols, having the amino and hydroxyl groups attached to contiguous carbon atoms, give an equivalent of formaldehyde on oxidation by periodate. After fractionation on a column of Dowex-2, followed by resolution on a column of buffered silica gel, they have been estimated quantitatively as periodate–formaldehyde (Rees, 1958). The four hydroxyamino acids, together with aspartic and glutamic acids, have been separated on a column of Dowex-50; the resolution of δ-hydroxy-α-aminovaleric acid (δ-α-HAV) from γ-hydroxy-β-aminobutyric acid (γ-β-HAB) is not at present satisfactory, but the latter hydroxyamino acid, as well as δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV), can be readily estimated without interference as periodate–formaldehyde for the same reason as β-amino alcohols. In the present work δ-α-HAV and γ-hydroxy-α-aminobutyric acid (γ-α-HAB), which do not give formaldehyde on oxidation by periodate, have not been determined directly. It was necessary also to study more closely the efficiency of the procedures in current use for esterifying proteins and this, in its turn, forced us to inquire into the ammonia N as well as the amide N of the native protein and ester preparations with which we were dealing. We believe that our studies in these two fields have revealed observations of importance in general protein chemistry as well as in our own particular problem, and for this reason we have preferred to present them in detached form as separate papers in the present series.

EXPERIMENTAL

Materials

Glycylglycyl-α-aminomyristic acid. We have to thank Professor Damodaran for the gift of a sample prepared by Abderhalden & Damodaran (1930).

Proteins. The preparations of insulin, β-lactoglobulin and lysozyme used are described elsewhere (Chibnall, Mangan & Rees, 1958*b*). In our earlier work the samples taken for reduction had been ground as finely as possible in an agate mortar. The present procedure is as follows. The protein (100 mg.) is dissolved in 0·033 N-HCl (3 ml.) and treated immediately with acetone (30 ml.). The precipitated protein is centrifuged off, washed twice with acetone, three times with ether (A.R. dried over sodium) and the product dried *in vacuo* at room temperature. Occasionally, as noted in the text, the drying stage has been omitted and the ether-washed material, after being treated twice with tetrahydrofuran, was put through the reduction procedure forthwith.

Protein methyl esters. These were prepared with methanolic 0·1 N-HCl at 25° for 24 hr. unless otherwise stated (Chibnall *et al.* 1958*c*).

O-*Acetylinsulin methyl ester.* Insulin (100 mg.) was dissolved in acetic acid saturated with urea (4 ml.) and treated with acetyl chloride (1·4 ml.) at room temperature for 1 hr. Acetone (55 ml.) was then added and the separated product centrifuged. The material was given ten washings with acetone (55 ml.) to remove urea. The procedure, which has been successfully applied also to serine and β-lactoglobulin, brings about exclusive *O*-acetylation and has the advantage that the reaction products remain soluble. The methyl ester was prepared with diazomethane, the reaction mixture being kept below pH 5.

Tetrahydrofuran. The commercial product was purified by the procedure of Bailey (1955) and stored over sodium in the dark. It was redistilled before use.

Lithium borohydride ($LiBH_4$). In our earlier work a standard solution prepared by the method of Bailey (1955) was used. More recently we have found that the commercial product (New Metals and Chemicals Ltd., London) contains much inert material, the reducing power as assessed by titration of the lithium with standard acid being only 70% and by iodate titration (Lyttle, Jensen & Struck, 1952) only 65% of theory. Purification was readily effected as follows. The material (5 g.) in a 250 ml. centrifuge bottle, was stirred with 200 ml. of ether (A.R., dried over sodium) and the mixture centrifuged. The clear ethereal solution was poured off and the residue treated with two further lots of ether in the same way. The ethereal extracts were collected and on removal of most of the solvent by evaporation the material crystallized in long needles. The treatment was repeated. The final product (about 2·5 g.) dissolved easily in cold tetrahydrofuran to give a clear colourless solution. The reducing power and titration agreed and were within 2% of theoretical. A stock solution (about 1·2 M) slowly deposits white insoluble material when kept in the dark, and it is necessary to centrifuge and re-check the reducing power immediately before use.

Procedures and methods

Reduction with $LiBH_4$. Experience has shown that for products which are insoluble in tetrahydrofuran, such as protein methyl esters, an eightfold molar excess of $LiBH_4$ is required to effect full reduction of the ester groups. Provision must also be made for the bound HCl and disulphide linkages present. It is convenient to adjust the volume of tetrahydrofuran to make an approx. 0·3 M-solution of reducing agent. The reaction vessel (10 cm. × 2 cm.) is fitted at the top with a three-necked multiple adapter, and a mercury-sealed stirrer is inserted through the centre socket. One of the side sockets carries a condenser with a soda–lime trap, and the other is stoppered. The protein or ester (0·1 g.) was placed in the open reaction vessel and dried *in vacuo* for 2 hr. at 50°. The adapter with its furnishings was next placed in position, and through the third socket was introduced first a standard $LiBH_4$ solution containing 13 mg. of reagent and then sufficient tetrahydrofuran to bring the total volume to 2 ml. The reaction mixture was then boiled gently (oil bath, 90°) for the required time, generally 6 hr. It was cooled to room temperature, a slight excess of methanolic HCl was added dropwise and the mixture was stirred for 1 hr. to ensure decomposition of excess of reagent and any borane complexes present. Acetone–ether (1:1, v/v; 8 ml.) was then added and the reduced protein removed by centrifuging. It was washed twice with acetone, once with ether, and then dried *in vacuo* over P_2O_5.

Hydrolysis. The products were boiled under reflux with 50–70 parts (v/w) of 10 N-HCl–formic acid (1:1, v/v) for 24 hr. and the excess of acid was removed in the usual way. Evaporations *in vacuo* were conducted below 45°.

Separation of the β-amino alcohol fraction. A column (10 cm. × 1 cm.) of Dowex-2 × 10 was regenerated with carbonate-free N-NaOH and then thoroughly washed with CO_2-free water. A sample containing approx. 30 mg. of the reduced protein hydrolysate in a volume of 1 ml. was transferred to the column, and the amino alcohol fraction eluted with CO_2-free water until 25 ml. had been collected.

β-Amino alcohols. These were estimated by periodate–formaldehyde by the procedure of Rees (1958).

δ-Hydroxy-γ-aminovaleric acid and γ-hydroxy-β-aminobutyric acid. δ-γ-HAV and γ-β-HAB were estimated by the procedure of Chibnall, Haselbach, Mangan & Rees (1958*a*).

RESULTS

Glycylglycyl-α-aminomyristic acid. The acid (100 mg.) was dissolved in tetrahydrofuran (7·6 ml.) containing 0·33 M-$LiBH_4$ and the solution boiled under reflux for 6 hr. The collected hydrolysis liquors were brought to 2·5 ml. and a sample of 1 ml. was passed through a column of Dowex-2. The amino alcohol fraction gave formaldehyde equivalent to 1·3 % of a glycyl residue as ethanolamine.

Insulin. The results given in Table 2 leave no doubt that what may be referred to, in light of Sanger's researches on insulin structure, as the adventitious amino alcohols ethanolamine, leucinol and tyrosinol, have in all three cases a common origin. Our earlier suggestion of an *N–O* acyl migration on esterification, followed by reductive cleavage of the resulting *O*-peptidyl ester, is clearly untenable, not only for the reasons mentioned briefly in the introduction, but also because it has been shown that this particular acyl shift is largely at the expense of the unique threonyl residue (Chibnall *et al.* 1958*c*), so that tyrosinol, from the sequence tyrosyl-threonyl, should have been produced to a greater extent than ethanolamine. Direct reductive cleavage of peptide bonds, as demonstrated by Crawhall & Elliott (1955), with lysozyme, must clearly be the major factor concerned. We have so far been unable to find conditions which will minimize this effect without at the same time impairing the reduction of the terminal ester group. Crawhall & Elliott found that the reductive cleavage with $LiBH_4$ at room temperature was negligible, even when the material concerned was soluble in the reaction mixture. This would be in agreement with the work of Bailey (1955) on simpler peptide systems. With insulin methyl ester, however, the reductive cleavage of peptide bonds runs more or less parallel with the

Table 2. *Amino alcohol analysis of insulin and various derivates after reduction with lithium borohydride*

Unless otherwise stated, all products were esterified with methanolic 0·1 N-HCl for 24 hr. and all reductions were made in boiling tetrahydrofuran for 6 hr. Materials: Expt. 1, insulin, finely ground crystals; Expt. 2, precipitated insulin (see text); Expt. 3, insulin ester reduced at 20° for 24 hr.; Expt. 4, insulin ester; Expt. 5, *O*-acetylinsulin esterified with diazomethane. Results are expressed as moles/mole of protein (5732 g.).

Expt. no.	Yield of amino alcohol					Total amino alcohol fraction from Dowex-2 (periodate–formaldehyde)	
	Leucinol*	Tyrosinol*	Alaninol*	Aspartidiol*	Ethanolamine†	Yield	Accounted for‡ (%)
1	—	—	0·017	—	0·013	0·04	103
2	0·048	0·023	0·156	—	0·161	0·61	85
3	0·03	0·011	0·655	0·026	0·135	0·95	97
4	0·1	0·028	1·111	0·08	0·19	1·7	92
5	0·111	0·033	1·077	0·04	0·16	1·50	97

* Corrected for 10 % loss on hydrolysis. † Gives 2 equiv. of formaldehyde.
‡ Based on the yields (uncorrected for loss on hydrolysis) of the amino alcohols concerned.

reduction of the *C*-terminal alanine ester group in spite of the complete insolubility of the ester in the cold reaction mixture (Table 2, no. 3).

The comparison between the reduction products of insulin and its methyl ester is set out in Table 3. It will be seen that the increase in alaninol represents in full measure the *C*-terminal alanine residue of the *B* chain. The increase in the γ-β-HAB is somewhat less than might have been expected for the *C*-terminal asparagine residue of the *A* chain, but this is due in part to the initial low amide N of the insulin ester (Chibnall *et al.* 1958*c*). The aspartidiol, which is a reduction product of the ester and not of the unesterified protein, represents a *C*-terminal aspartic acid residue and this, in its turn, must represent an asparagine residue which has lost its amide N. All the remaining products listed in Table 3 represent adventitious reductive cleavage of peptide linkages and the various differences between the two sets of data are surprisingly small. It is interesting to note that residues of glutamine, giving rise to δ-γ-HAV, are particularly labile, whereas those of glutamic acid, which with the protein ester would give rise to glutamidiol, appear to be quite stable.

Lysozyme. Three different reduction treatments were applied to the ester, and the most drastic of these to the protein. The reduction products given on hydrolysis are listed in Table 4. In agreement with Crawhall & Elliott (1955) there is evidence of random reductive cleavage of peptide bonds, and in addition to the amino alcohols quoted, tyrosinol, valinol and serinol were present in amounts too small for inclusion in the table. Residues of glutamine and asparagine were labile, as shown by the production of δ-γ-HAV and γ-β-HAB respectively, but the absence of aspartidiol indicates that the free aspartic acid residues (Chibnall *et al.* 1958*a*) were untouched.

Thompson (1952) and Harris (1952), using carboxypeptidase, found that leucine occupies the *C*-terminal position. Our findings are in partial agreement with this, because leucinol is the only reduction product which shows a substantial increase as between ester and protein. Yet under all three of our reduction treatments, the mildest of which (Table 4, no. 1) has given full reduction of the aspartyl residues (Chibnall *et al.* 1958*a*), the amount of leucinol produced does not exceed 0·5 mole/mole, allowing for the protein control. Ohno (1953), using hydrazinolysis, reported one *C*-terminal leucine residue/mole, but his experimental data are subject to a very large correction for losses.

β-Lactoglobulin. This protein has a larger molecular weight than lysozyme and one peptide bond represents only 0·243% of the total protein N. A small degree of peptide cleavage may thus assume inflated proportions when measured in terms of groups/mole. The data given in Table 5

Table 3. *C-Terminal residues in insulin*

Insulin was esterified with methanolic 0·1 N-HCl for 24 hr. Insulin and ester were reduced with $LiBH_4$ in boiling tetrahydrofuran for 6 hr. Results are expressed as moles/mole of protein (5732 g.).

	Yield of amino alcohol or hydroxyamino acid						
Material	Leucinol	Tyrosinol	Alaninol	Aspartidiol	Ethanolamine	γ-β-HAB	δ-γ-HAV
Insulin methyl ester	0·1	0·027	1·110	0·08	0·19	0·89	0·18
Insulin	0·048	0·023	0·156	—	0·161	0·08	0·16
Difference	0·052	0·004	0·955	0·08	0·029	0·81	0·02

Table 4. *Reduction products of lysozyme and its methyl ester*

Methyl ester was prepared with methanolic 0·1 N-HCl at 25° for 24 hr. Treatment before reduction: no. 1, material was washed successively with acetone and ether, and dried *in vacuo*; no. 2, material was washed successively with acetone, ether and tetrahydrofuran and reduced forthwith (see text). Results are expressed as moles/mole of protein (14 700 g.)

			Yield of amino alcohol or hydroxyamino acid					
Material	Treatment	Time of reduction (hr.)	Amino alcohol fraction (Dowex-2) periodate–formaldehyde yield	Leucinol	Alaninol	Ethanolamine	γ-β-HAB	δ-γ-HAV
Ester	No. 1	6	1·68	0·53	—	—	—	—
Ester	No. 2	2	2·88	0·57	0·25	0·71	—	—
Ester	No. 2	6	3·48	0·54	0·6	0·75	0·86	1·03
Protein	No. 2	6	2·58	0·1	0·6	0·63	0·67	1·0

Table 5. *Reduction products of β-lactoglobulin and its methyl ester*

Methyl ester was prepared with methanolic 0·1 N-HCl at 25° for 24 hr. Results are expressed as moles/mole of protein (37 000 g.).

Material	Amino alcohol fraction (Dowex-2) periodate–formaldehyde yield	Yield of amino alcohol or hydroxyamino acid					
		Leucinol	Tyrosinol	Alaninol	Ethanolamine	γ-β-HAB	δ-γ-HAV
Ester	9·9	1·49	0·63	5·50	0·82	0·9	1·5
Protein	4·0	0·34	0·3	1·60	0·52	0·8	1·7
Difference	5·9	1·15	0·33	3·9	0·3	0·1	−0·2

demonstrate that fairly widespread peptide cleavage has occurred and, as with lysozyme, the analysis for amino alcohols showed the presence of valinol, serinol and the two diols, but in amounts too small for inclusion in the table. Comparison of results for the ester and protein shows an increase of 1 mole of leucinol and 4 moles of alaninol/mole of protein, suggesting that the molecule consists of at least five peptide chains, one of which has a *C*-terminal leucine residue and the other four have alanine residues. This finding is in contrast with that of Porter (1948), who was able to recognize no more than three *N*-terminal leucine residues by the DNP procedure of Sanger (1945), and is referred to again later.

DISCUSSION

The observations recorded above show that facile and quantitative reduction of the esterified carboxyl groups of proteins can be obtained under relatively mild conditions with lithium borohydride. The results, however, leave no doubt that the issue is complicated by the reductive fission of peptide bonds, as suggested by Crawhall & Elliott (1955), and that the contrary assertions of Grassmann, Hörmann & Endres (1953, 1955) and of Grassmann, Strobel, Hannig & Deffner-Plockl (1956) were based on inadequate experimental evidence. It is true that as a side reaction in such a major operation the effect is small (Table 6), but as has already been emphasized for β-lactoglobulin, it can be sufficiently large to cast doubt on any evaluation of the end groups in a protein of molecular weight greater than that, say, of insulin. Moreover, the effect depends in part on the state of dispersion of the product at the reduction stage, and it might be argued that with insulin (Table 3), and perhaps also with lysozyme (Table 4), the two contrasted products have been more closely balanced in this respect than was apparently the case with β-lactoglobulin (Table 5). The data given in the tables are presented with the object of illustrating these and many other points discussed in the text, and too much significance should not be attached to the numerical value of the results deduced.

Table 6. *Reductive cleavage of peptide bonds by lithium borohydride*

Values for N are computed from the sum of the formaldehyde equivalent of δ-γ-HAV, γ-β-HAB and amino alcohol fraction (Dowex-2, less allowance for excess given by ethanolamine).

Product	N (% total N)	Peptide bonds cloven/mole
Polyglutamic acid from *B. subtilis*	0	0
Glycylglycyl-α-amino-myristic acid	0·65	0·013
Insulin	1·07	0·69
β-Lactoglobulin	1·51	6·0
Lysozyme	1·84	3·62

In agreement with Crawhall & Elliott we find that the reductive fission by lithium borohydride is selective. α-Glutamyl and α-aspartyl residues in proteins are remarkably resistant, as also are the γ-glutamyl residues in the bacterial polypeptide from *Bacillus subtilis* (Chibnall, Rees & Richards, 1958), suggesting that it is the free carboxyl group which confers protection. Glutamine and asparagine residues, on the contrary, are susceptible, giving rise to δ-γ-HAV and γ-β-HAB respectively. In all the three proteins examined the amount of each hydroxyamino acid produced was the same for the protein as for the ester, so that their origin cannot be ascribed to the presence of γ- or β-linkages respectively in the peptide chains. The observation needs emphasis because γ-linkages have been suggested by Fromageot & Jutisz (1953) on no more evidence than the detection of δ-γ-HAV in hydrolysates of proteins reduced with lithium aluminium hydride ($LiAlH_4$) (cf. Haurowitz & Horowitz, 1956).

It will thus be seen that in practice the procedure under review for determining *C*-terminal residues in proteins, like all others based on chemical principles which have been suggested in recent years, has been found wanting. If applied with due regard to its limitations it may well yield valuable information with polypeptides of molecular weight of the order 10 000 or less, but the main problem of determining by chemical means the *C*-terminal

residues in proteins, the need for which was emphasized in the introduction, is still unsolved and remains a challenge to the analyst.

SUMMARY

1. The carboxyl groups of *C*-terminal residues in proteins, after preliminary esterification, can be reduced with lithium borohydride, and on subsequent acid hydrolysis the modified residues are present in the hydrolysate as amino alcohols or hydroxyamino acids as the case may be.

2. The suitability of the procedure for determining the *C*-terminal residues in proteins has been explored, and in agreement with Crawhall & Elliott (1955), it was found that the issue was complicated by the simultaneous reductive cleavage of peptide bonds which occurs, under the conditions chosen, to the extent of 1–2% of the total peptide bonds.

3. Data are presented showing that with a protein of low molecular weight such as insulin (5732) the interference is not serious and a satisfactory determination of the *C*-terminal residues can be made. With a protein of much higher molecular weight such as β-lactoglobulin (37 000), however, the interference is serious.

4. The procedure is not recommended as a reliable one for proteins but it may be of use with peptides of low molecular weight.

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Studies on the Amide and *C*-Terminal Residues in Proteins

2. THE AMMONIA NITROGEN AND AMIDE NITROGEN OF VARIOUS NATIVE PROTEIN PREPARATIONS

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In all our previous work which called for a determination of amide N, the protein preparation used for the purpose had been coagulated by heat and washed free from inorganic salts, so that the ammonia N content was negligible and a direct determination of the amide N permissive. Such heat-coagulated products, however, do not readily esterify, and in the present investigation it has been necessary to use the native crystalline material. This may have been fractionated from natural sources by reagents other than ammonium salts, and the product thus obtained may have been dialysed for some days against distilled water; even so, we have been surprised to find that it can

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contain ammonia N. The amount present may be small, yet in a protein of high molecular weight it may be equivalent to one or more groups of amide N per molecule, thus making less definite the estimation of the latter factor. A procedure for determining this ammonia N, which depends on the fact that the protein can be precipitated from methanolic hydrochloric acid by ether, while the ammonium salts remain in solution, is described in the present paper. It has been applied to native samples of several different proteins and, allowing for the ammonia N thus determined, it has been shown, for instance, that the corrected value for the amide N of native β-lactoglobulin is in very close agreement with that recorded for the heat-coagulated product, and that all the samples of crystalline ox insulin examined, except that prepared by countercurrent distribution, have low corrected values for amide N, suggesting loss in manufacture.

EXPERIMENTAL

Materials

Ox insulin. (1) Two samples of crystalline Zn insulin were obtained from Boots Pure Drug Co. Ltd., Nottingham (batch no. 9633B and 9011G). They had been prepared under identical conditions and recrystallized six times from a citrate buffer. Batch 9633B contained (moisture-free) 15·76% N (micro-Kjeldahl) and 15·83% N (micro-Dumas). (2) A sample for which we thank Dr L. C. Craig; it was the '*A* peak' material obtained by countercurrent extraction of a commercial product (Harfenist & Craig, 1952). (3) A sample for which we thank Dr R. R. Porter; it had been purified by chromatography (Porter, 1953).

β-Lactoglobulin. Samples *A* and *B* were prepared by the procedure of Palmer (1934), $(NH_4)_2SO_4$ and Na_2SO_4 fractionation respectively being used. The twice-recrystallised products had been dialysed against distilled water for 3 days.

β_1-Lactoglobulin. We have to thank Dr T. L. McMeekin for a sample of crystalline material prepared by the method of Polis, Schmukler, Custer & McMeekin (1950).

Lysozyme. Prepared from egg-white (Armour, no. 20793).

Methods

Total nitrogen. This was estimated by the procedure of Chibnall, Rees & Williams (1943).

Ammonia nitrogen. This was estimated by the ninhydrin method of Moore & Stein (1954), with cyanide (Troll & Cannan, 1953; Yemm & Cocking, 1955) in place of hydrindantin. The colour intensity with NH_3 was affected by the presence of salts, and to overcome this a sodium acetate buffer (5M) was used. The colour yield was then reproducible and 80% of that obtained from leucine. Small quantities of the reagent can readily be made up as required.

The procedure was as follows. Sodium acetate buffer, pH 5·5 (5M), was stored at 37°. To a sample containing approx. 1·5–5 μg. of ammonia N in a volume of 2 ml. was added 1 ml. of a solution made from buffer (23 ml.), 0·01M-KCN (2 ml.) and methyl Cellosolve (2-methoxyethanol) (75 ml.) followed by 10% (w/v) ninhydrin in methyl Cellosolve (0·2 ml.). After shaking, the colour was developed, diluted and estimated in the usual way against a standard curve. In some of the studies on β-lactoglobulin we used the hypochlorite procedure of Van Slyke & Hiller (1933), as modified by Russell (1944). It is satisfactory, but the sensitivity of the colour developed is considerably less than with ninhydrin.

Amide nitrogen. The protein was hydrolysed by boiling with 2N-HCl (3 mg./ml.) for 3 hr. The subsequent treatment depended on the amount of material concerned. If this was in excess of 0·1 g., the hydrolysate was brought to pH 4 (bromocresol green) with 4·8N-NaOH, run into a micro-Kjeldahl apparatus and made alkaline by the addition of 10 ml. of 0·3M-Na_2HPO_4–NaOH, pH 11, buffer solution. The NH_3 was then distilled over into 0·0143N-HCl. If the quantity of protein was of the order of 5–10 mg. suitable samples of the hydrolysate were taken and the Conway technique was employed, the NH_3 being estimated with ninhydrin. The value found (Table 1) is a measure of (amide N + ammonia N), and to obtain the first the second must be subtracted from the total.

Estimation of ammonia nitrogen in proteins. The protein (10 mg.) was dissolved in 1 ml. of methanolic 0·033N-HCl and at once precipitated by adding ether (2 ml.). It was removed by centrifuging and the operation was twice repeated. The collected liquors were then evaporated to dryness *in vacuo* to remove solvents, and ammonia N was determined with ninhydrin after distillation in the Conway

Table 1. *Amide nitrogen of various proteins*

Protein	Sample, literature reference or source	Amide N (found) % total N	Ammonia N (% total N)	Amide N (corrected) % total N	Amide N (corrected) Groups/mole
β-Lactoglobulin	*A*	7·06	0·26	6·80	28·04
	B	6·88	0·05	6·83	28·16
	Rees (1946)	6·77	—	6·77	27·93
	Cannan *et al.* (1942)	6·79	—	6·79	28·0
	Mean	—	—	6·80	28·03
β_1-Lactoglobulin	Dr T. L. McMeekin	7·32	0·60	6·72	27·73
Insulin	Boots: (9633B, 9011G)	9·08	0·16	8·92	5·81
	Dr L. C. Craig (fraction *A*)	9·38	0·14	9·24	6·0
	Dr R. R. Porter	9·19	0·85	8·34	5·42
Lysozyme	Armour 20793	9·19	0·05	9·14	17·9

apparatus. To ascertain whether the treatment completely removed all the NH_4^+ ions a sample of insulin containing 1·825 mg. of N was passed through it, the ammonia N content of the three supernatant liquors being separately determined. The amounts found were 2·34, 0·46 and 0·13 μg. of ammonia N, equivalent to 0·128, 0·025 and 0·007% total protein N respectively, showing that the treatment was suitable for the purpose. The residual solubility of the protein in the methanolic HCl–ether mixture was of the order of 1%. As a check on the Conway procedure a sample of 180 mg. of Boots insulin was given the standard treatment and the collected liquors were reduced to a small volume *in vacuo*. Appropriate portions were then taken for the estimation of ammonia N by the ninhydrin–Conway procedure and by distillation under micro-Kjeldahl conditions. The results were in excellent agreement.

RESULTS AND DISCUSSION

β-Lactoglobulin. The data given in Table 1 present some interesting contrasts. The two samples of native crystalline material, which had such very different contents of ammonia N, gave corrected values for amide N which were not only in excellent agreement with each other, but also with those obtained in two earlier studies in which heat-coagulated and washed products had been used. Cannan, Palmer & Kibrick (1942) hydrolysed the protein with N-HCl at 100°, and Rees (1946) employed 10N-HCl at 37° (Gordon, Martin & Synge, 1941); in each case the amide N was determined at successive intervals of time and extrapolated to zero. If the protein N is taken as 15·6% (Chibnall *et al.* 1943) and the mean value for the amide N as 6·50% of the total N, then 28 groups of amide N/mole of protein leads to a molecular weight of 36 960, which is in good agreement with recent deductions based on physical measurements. One group of amide N is thus equivalent to 0·243% of the total N, and it will be observed that the ammonia N of sample *A* is in excess of this value.

β_1-Lactoglobulin. The molecular weight of this protein has not yet been determined and we have assumed it to be the same as that of β-lactoglobulin itself; the data given in Table 1 call for no comment.

Insulin. According to Sanger, Thompson & Tuppy (1952) the minimum mol.wt. of ox insulin is 5732 and the amide N is 9·24% of the total N. The two commercial samples used in the present work contained 9·08% amide N without allowance being made for any ammonia N present, and it was, in part, upon this uncorrected value that our original estimate of the minimum mol.wt. of insulin (12 000 or 6000) was based (Chibnall, 1946). Harfenist & Craig (1952) have since shown by countercurrent distribution that commercial insulin can be separated into two fractions, an '*A* peak' containing about six groups and a '*B* peak' with only about five groups of amide N respectively (Harfenist, 1953).

Dr Craig (private communication) informed us that our sample of Boots insulin (batch no. 9633B) contained about 85–90% of the '*A* peak' and 5–8% of the '*B* peak' material. The '*A* peak' material provided by Dr Craig (origin not stated) was the only sample of insulin we have handled during the present research which gave a (corrected) theoretical value for amide N. Harfenist & Craig suggested that their '*B* peak' material, of lower amide content, may have been an artifact of hydrolysis during manufacture of the insulin: we are in agreement with this view and shall present further data in a later paper. The preparation of purified insulin by the chromatographic method of Porter (1953) has clearly not differentiated between the *A* and *B* peak material of Harfenist & Craig. In spite of the fact that metallic salts were used in the fractionation, the final product contained a high proportion of ammonia N. Indeed, the uncorrected value for amide N given in Table 1 illustrates the danger of omitting an estimation of ammonia N when the amide N of a protein is in question.

Lysozyme (mol.wt. 14 700). The amide N given in Table 1 is in agreement with that of Lewis, Snell, Hirschmann & Fraenkel-Conrat (1950).

SUMMARY

1. Attention is drawn to the need to ascertain the free ammonia content of native protein preparations as a factor in the determination of the amide nitrogen.

2. A sample of ox insulin prepared by countercurrent distribution had a theoretical amide nitrogen of six groups/mole (5732 g.); all other samples examined had low values, which suggested loss during manufacture from the pancreas.

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Studies on the Amide and *C*-Terminal Residues in Proteins

3. THE ESTERIFICATION OF PROTEINS

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(*Received* 19 *June* 1957)

The carboxyl groups of a protein can be esterified with diazomethane, but more conveniently with methanolic hydrochloric acid. In the present research, however, neither reagent has proved to be entirely satisfactory, for in our hands the first has not given full esterification and the second would do this only at the expense of the amide N, which we were particularly anxious to keep intact. Fraenkel-Conrat & Olcott (1945) were the first to show that the concentration of mineral acid (0·02 to 0·1N) required to catalyse the reaction with methanol was very much less than previous workers had employed for the purpose. They claimed that 97% of the carboxyl groups of polyglutamic acid from *Bacillus subtilis* could be esterified by treatment with methanolic 0·05N-HCl at 22–24° for 24 hr. and that several proteins, including insulin, were fully esterified under similar conditions in the presence of 0·1N-HCl. They mentioned that there was no loss of amide N under such treatment, though data in support of the assertion were not presented. Mommaerts & Neurath (1950) repeated the experiments with insulin, and confirmed that full esterification was apparently achieved with methanolic 0·1N-HCl at 25° in 24 hr. They claimed that the ammonia liberated under these conditions amounted to not more than 2% of the amide N.

Our own observations do not support the contention that 0·1N-HCl can be used as a catalyst in the esterification of proteins in this way without loss of amide groups. With insulin the ammonia produced in 24 hr. at 25° represents about 6·6% of the amide N, a value much greater than the above-mentioned workers seem to have suspected. Lowering the concentration of hydrochloric acid spares the amide N but unfortunately full esterification is not then achieved. We have thus been obliged to prepare the ester with methanolic 0·1N-HCl, knowing that when this is used subsequently to determine *inter alia* the amide distribution of the protein, we had already set a limit to the accuracy with which this could be done. Another interesting side-reaction of protein esterification has been traced to *N–O* acyl migration in serine and particularly threonine residues.

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EXPERIMENTAL

Materials

Protein samples, and methods for determining N, ammonia N and amide N were described in the preceding paper (Chibnall, Mangan & Rees, 1958*b*).

Methoxyl. The Zeisel procedure of Pregl (1937) was used. Estimations on 20–30 mg. samples agreed to within 2%.

Methanol. This was boiled under reflux for some hours with Mg turnings and then distilled.

Methanolic hydrochloric acid. An approx. 2N-solution was prepared with dry HCl gas and stored at −15°. As the acidity falls on keeping it is necessary to titrate a sample immediately before use.

Methods

Estimation of the extent of esterification. The extent of esterification of the total free carboxyl groups was followed by determination of methoxyl. As β-lactoglobulin contains methionine, which yields methyl iodide on treatment with HI, the (apparent) methoxyl content of the protein itself was subtracted from that given by the ester. In studies dealing with the relative rates of esterification of glutamyl and asparagyl residues (Tables 1 and 4) the number of the respective residues esterified was computed from an analysis of the reduced protein hydrolysate (see Chibnall, Haselbach, Mangan & Rees, 1958*a*). Amide hydrolysis during esterification was followed by applying to the ester, while still in solution, the procedure for the

estimation of ammonia N in proteins described in the previous paper (Chibnall *et al.* 1958*b*).

Esterification with methanolic hydrochloric acid. The protein (0·25 g.) was placed in a flask (50 ml.) and dried overnight *in vacuo* at room temperature over P_2O_5 and NaOH. Methanol (20 ml.) was added, followed by methanolic HCl drop by drop until the protein dissolved. Sufficient excess of both methanolic HCl and methanol was then added to bring the final volume to 25 ml. and the acid concentration to that required. The protein was sometimes partly precipitated, especially with the stronger acid catalyst, but always as a fine emulsion, and mechanical stirring during the incubation period was unnecessary. The flask was then immersed in a water bath at 25° and shaken slowly for the prescribed period. About 60 ml. of ether was next added and after standing for a short time the precipitated ester hydrochloride was removed by centrifuging, washed twice with ether (50 ml.), dried *in vacuo* over P_2O_5 and NaOH and then allowed to equilibrate in the air at room temperature.

Esterification with diazomethane. To suppress ionization of the free carboxyl groups it is necessary to employ an acid medium and 85% (v/v) ethanolic 0·03N-HCl is a suitable one for insulin and lactoglobulin as both proteins are soluble in it. The reagent rapidly neutralizes the HCl, and as the protein becomes progressively more basic during esterification the pH of the medium will rise, favouring both O and N methylation, unless controlled by the addition of more acid. In the early stages of the work an ethereal solution of diazomethane was added in small portions at a time, alternately with drops of HCl, until the yellow colour of the chilled protein solution was permanent for at least 5 min. As an improvement the pH was controlled with a spot indicator (bromocresol purple). Under such conditions not more than three of the four ω-carboxyl groups of glutamic acid in insulin were esterified (Chibnall & Rees, 1952, 1953). More complete esterification can be effected in the following way. The reaction vessel is equipped with a glass electrode–calomel cell assembly, a stirrer and two burettes with long capillary tips, one for the addition of the cooled ethereal reagent and the other for 6N-HCl. The vessel, containing a 1% solution of the protein in the above-mentioned solvent, was stood in a freezing mixture. Ethereal diazomethane (a twofold excess allowing for the HCl as well as the protein carboxyl groups) was then run in slowly with stirring, the (apparent) pH being controlled between 4 and 5 by the necessary addition of HCl. At the end of the reaction a slight excess of HCl was added and the ester hydrochloride precipitated with 8–10 volumes of ether, washed twice with ether and dried *in vacuo* over P_2O_5 and NaOH. According to our experience such products often contain a small amount of trimethylamine, which is not readily removed and can lead to high values for amide N.

RESULTS AND DISCUSSION

The data given in Table 1 show that full esterification of β-lactoglobulin with diazomethane had not been achieved under any of the conditions employed. Our results with insulin were similar, and an earlier claim (Chibnall & Rees, 1953) that full esterification was possible provided that the reaction mixtures was kept below pH 5 was based on a molecular weight of 6000 and not on the present accepted value of 5732. With methanolic 0·1N-HCl the esterification is complete in 24 hr. at 25° (Table 2), but, as has been mentioned earlier, appreciable alcoholysis of the amide groups occurs. As these observations on the lability of the amide N are in partial conflict with those of Mommaerts & Neurath (1950) the full protocol for one experiment on insulin is given in Table 3. For the studies reported in other papers of the series esterification with methanolic 0·1N-HCl has been found to be the only workable compromise for our purpose.

The data given in Table 4 illustrate the progress of the esterification of the various free carboxyl groups in insulin and β-lactoglobulin. The fact that the theoretical maximum is exceeded in all cases is due to amide hydrolysis. It is to be noted that the α-carboxyl of the *C*-terminal asparagine residue in insulin esterifies at a faster rate than the γ-carboxyl groups of the glutamyl residues, whereas in β-lactoglobulin the latter react rapidly and at a rate which exceeds that of the α-carboxyls of the aspartyl residues.

Acyl migration on esterification

A phenomenon accompanying the esterification of insulin and β-lactoglobulin in methanolic hydrochloric acid is an increase in free amino N (Van Slyke). In a previous communication (Chibnall & Rees, 1953) we had discussed the possibility that this might be due to an *N–O* acyl shift at serine and threonine residues to give the corresponding *O*-peptidyl ester and a free amino group, in keeping with the earlier findings of Desnuelle & Casal (1948) and of Elliott (1952), who had shown that such a change could take place under the influence of strong mineral acid. Under our conditions the increase in amino N is much less than Elliott observed, but it is reversible in alkaline solution (Tables 5 and 6) and the data presented in Table 7 show that serine and threonine residues are concerned in the reaction. In insulin, somewhat surprisingly, the threonine residue is the more labile. When the ester is made with diazomethane, the pH being kept below 5 to prevent *N*-methylation, there is no appreciable increase in amino N, suggesting that it is the catalytic action of the hydrochloric acid which promotes the acyl shift and not the suppression of free carboxyl groups as mentioned in our earlier communication. That such a shift is a sensitive one and prone to reversal, as though it involved a state of molecular strain, would follow from the observation that when a sample of insulin ester hydrochloride was analysed some months after preparation, the amino N had fallen from an initial 3·6 to 3·2 groups/mole.

Table 1. *Esterification of β-lactoglobulin with diazomethane*

Protein preparation *A*: no. 1, esterified without definite pH control; no. 2, reaction mixture was maintained below pH 7; no. 3, reaction mixture was maintained between pH 4 and 5. For comment on the number of residues esterified see text.

	Glutamyl residues		Aspartyl residues	
No.	Residues esterified/mole of protein (37 000 g.) (max. 32)	Esterification (%)	Residues esterified/mole of protein (37 000 g.) (max. 21)	Esterification (%)
1	27·5	86·0	17·4	82·7
2	32·4	101·2	16·2	77·2
3	30·6	95·6	19·5	92·8

Table 2. *Extent of esterification attained with various strengths of methanolic hydrochloric acid*

Insulin: Boots, 9011 G; amide N and total carboxyl groups are 6 and 6 groups/mole of protein (5732 g.) respectively. β-Lactoglobulin; sample *A*; amide N and total carboxyl groups are taken as 28 and 56 groups/mole of protein (37 000 g.) respectively (Chibnall *et al.* 1958*a*). Results are expressed as groups/mole of protein.

	Insulin			β-Lactoglobulin		
		Carboxyl groups esterified			Carboxyl groups esterified	
Material and normality of methanolic HCl used for esterification	Amide N (found)	Max. possible	Found (methoxyl)	Amide N (found)	Max. possible	Found (methoxyl)
Protein	5·82	6·18	—	28	56·0	—
Ester, 0·025 N	5·65	6·35	5·43	—	—	—
Ester, 0·033 N	5·60	6·40	5·8	27·1	56·9	44·8
Ester, 0·05 N	5·57	6·43	—	26·5	57·5	55·5
Ester, 0·1 N	5·43	6·57	6·4	26·0	58·0	59·1

Table 3. *Hydrolysis of the amide group of insulin during esterification with methanolic hydrochloric acid*

		Total ammonia N			Amide N	
Material	Total N in sample (mg.)	mg.	% protein N	Decrease in amide N (% protein N)	% protein N	Groups/mole of protein (5732 g.)
Insulin	36·5	0·0586	0·16	—	8·92	5·80
Ester (0·025 N-HCl)	36·08	0·1015	0·28	0·12	8·80	5·72
Ester (0·033 N-HCl)	36·08	0·1170	0·32	0·16	8·76	5·69
Ester (0·05 N-HCl)	36·24	0·1595	0·44	0·28	8·64	5·61
Ester (0·1 N-HCl)	36·17	0·2781	0·77	0·61	8·31	5·40

Table 4. *Rate of esterification of glutamyl, aspartyl and C-terminal asparagine residues*

Insulin, Boots (9011 G); β-lactoglobulin, sample *A*; esterified for the period stated in methanolic 0·1 N-HCl at 25°. For comment on the number of residues esterified, see text.

	Insulin				β-Lactoglobulin			
	Glutamyl residues		*C*-terminal asparagine residues		Glutamyl residues		Aspartyl residues	
Time of esterification (hr.)	Esterified residues/mole of protein (5732 g.)	Esterification (%)	Esterified residues/mole of protein (5732 g.)	Esterification (%)	Esterified residues/mole of protein (37 000 g.)	Esterification (%)	Esterified residues/mole of protein (37 000 g.)	Esterification
0·5	1·9	47·5	0·65	65·0	22·1	69·1	7·3	34·7
1·0	—	—	—	—	27·7	86·6	8·1	38·6
2·2	2·6	65·0	0·9	90·0	—	—	—	—
4·0	3·1	77·5	1·05	105·0	33·0	103·2	15·4	73·3
24·0	4·1	102·5	1·1	110·0	34·8	108·7	21·5	102·5

Table 5. *Increase in amino nitrogen on esterification of β-lactoglobulin and its reversal on treating the ester with dilute alkali*

Procedures before Van Slyke analysis: 1, sample *A*, esterified in methanolic 0·1 N-HCl; 2, sample *B*, esterified as above for 24 hr.; 45·2 mg. of ester was dissolved in 15 ml. of 0·01 N-sodium borate buffer (pH 9·2) at 20·3° to give pH 8·9; 5 ml. portions were taken for analysis.

Material	Procedure	Time of esterification (hr.)	Time of treatment in alkali (hr.)	Amino N: % protein N	Amino N: Groups/mole of protein (37 000 g.)	Amino N: Increase or decrease
Protein *A*	1	0	—	7·10	29·3	—
		0·5	—	7·43	30·6	+1·3
		4·0	—	7·85	32·4	+3·1
		24·0	—	7·99	32·9	+3·6
Ester of protein *B*	2	—	0	8·03	33·1	—
		—	1·0	7·84	32·3	−0·8
		—	4·0	7·29	30·0	−3·1
Protein *B*	—	—	—	6·98	28·8	—

Table 6. *Increase in amino nitrogen on esterification of insulin and its reversal on treating the ester in dilute alkali*

Procedure before Van Slyke analysis: 1, Insulin (Boots, 9011 G) or its methyl ester hydrochloride was dissolved in water; 2, insulin was dissolved in dil. HCl; 3, methyl ester hydrochloride (40 mg.) was suspended in 0·2 ml. of 0·3 N-NaOH and kept at 0° for the required time; the product was then dissolved in a minimum excess of dil. HCl; 4, methyl ester hydrochloride (110 mg.) was dissolved in 10 ml. of aq. methanolic (85 %) 0·3 N-NaOH and kept at 0° for 1 hr. The product was then precipitated with excess of acetone, dried with ether and dissolved in a minimum excess of dil. HCl.

Material	Procedure	Time of esterification (hr.)	Time of treatment in alkali (min.)	Amino N: Temperature	Amino N: Reaction time (min.)	Amino N: Groups/mole of protein (5732 g.)	Amino N: Increase or decrease
Insulin	1	—	—	20·3°	15	2·87	—
Insulin hydrochloride	2	—	—	20·3	15	2·96	+0·09
Ester hydrochloride	1	0·5	—	20·3	15	3·10	+0·23
Ester hydrochloride	1	2·0	—	20·3	15	3·19	+0·32
Ester hydrochloride	1	4·0	—	20·3	15	3·25	+0·38
Ester hydrochloride	1	24·0	—	20·3	15	3·60	+0·73
Ester hydrochloride	1	24·0	—	22·5	11	3·65	—
Ester hydrochloride	3	24·0	5·0	22·5	11	3·30	−0·35
Ester hydrochloride	3	24·0	45·0	22·5	11	3·20	−0·45
Ester hydrochloride	4	24·0	60·0	21	11	2·90	−0·75

Table 7. *Serine and threonine analyses illustrating acyl migration on esterification of insulin in methanolic 0·1 N-hydrochloric acid*

The products were deaminated (Peters & Van Slyke, 1932), and then analysed on a column of Dowex-50 with ninhydrin (Moore & Stein, 1954). 1, Insulin; 2, insulin methyl ester (24 hr. at 25°); 3, insulin methyl ester treated with alkali as described in Table 6, procedure 3. In calculating the results a loss of 3 % total N on deamination has been assumed. Serine and threonine values have been corrected for losses on hydrolysis (Rees, 1946). Results are expressed as residues/mole of protein (5732 g.).

Products	Yield of amino acid: Aspartic acid	Threonine	Serine	Glutamic acid
1	2·97	1·02	3·12	6·98
2	2·93	0·73	2·95	6·90
3	2·96	0·91	3·14	7·02

SUMMARY

1. Methods of esterifying proteins have been investigated in detail.

2. It has not been found possible to esterify a protein fully with diazomethane, even when the reaction is allowed to proceed below pH 5.

3. Full esterification can be achieved with methanolic 0·1 N-HCl in 24 hr., in confirmation of the results of earlier workers. The hydrolytic loss of amide nitrogen is, however, much greater than has hitherto been reported.

4. Data are presented to show the relative rates of esterification of aspartyl, asparaginyl and glutamyl residues in insulin and β-lactoglobulin.

5. There is an increase of amino nitrogen (Van Slyke) on esterification of proteins with methanolic 0·1 N-HCl. The effect is reversed in an alkaline

medium and has been shown to be due to an *N–O* acyl shift in serine and threonine residues.

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Studies on the Amide and *C*-Terminal Residues in Proteins

4. SEPARATION AND QUANTITATIVE DETERMINATION OF β-AMINO ALCOHOLS

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The procedure for characterization of the *C*-terminal residues in proteins which was described in the first paper of this series (Chibnall & Rees, 1958) called for a method for the separation and estimation of the β-amino alcohols present in the hydrolysate of the esterified and reduced protein. At the time the investigation was started no such method was available, although subsequently the isolation and chromatographic separation of dinitrophenylamino alcohols has been described by a number of workers (Luck & Wilcox, 1953; Jutisz, Privat de Garilhe, Suquet & Fromageot, 1954; Jatzkewitz & Tam, 1954; Grassmann, Hörmann & Endres, 1954; Crawhall & Elliott, 1955).

A partition column of silica gel on gradient elution with ethanol–chloroform has been found to effect a good separation of such of the β-amino alcohols as were encountered when dealing with insulin, lysozyme and β-lactoglobulin. The identities of the products giving rise to the effluent peaks were checked by paper chromatography, and these products could be quantitatively estimated as formaldehyde after periodate oxidation. Because of the limitations to which the reductive procedure is prone (Chibnall & Rees, 1958), no attempt has yet been made to test the behaviour of such other β-amino alcohols as might occur, but there is no doubt that the method of separation is capable of further development. Early experiments on the use of Dowex-50 were not continued because of low recoveries; paper chromatography was also tried but had to be abandoned because of persistent streaking due to traces of inorganic salts and low recoveries.

* Member of the Scientific Staff of the Agricultural Research Council.

MATERIALS AND METHODS

β-Amino alcohols. Alaninol, aspartidiol and glutamidiol were prepared by reduction of the corresponding amino acid ester hydrochloride. They were purified by distillation *in vacuo*. The other β-amino alcohols used were obtained from L. Light and Co., Wraysbury. As most of the products are oils, aqueous solutions were prepared and standardized for total N and formaldehyde given on periodate oxidation. Suitable portions containing 1–3 μmoles of each β-amino alcohol were then taken for chromatography.

Buffer solution (pH 4·85). $Na_2HPO_4,12H_2O$ (A.R., 184·5 g.) and citric acid monohydrate (A.R., 51 g.) were dissolved in water and made up to 1 l. Chloroform (1–2 ml.) was added as preservative.

Solvent A. Lower phase of the mixture: buffer solution–ethanol–chloroform (1:1:1).

Solvent B. Upper phase of the mixture: buffer solution–ethanol–chloroform (1:2:1). The mixtures were shaken in separating funnels and left overnight at 27·5±1° to equilibrate; the solvent phases were filtered before use.

Column chromatography

Preparation of column. Silica gel (5 g., Tristram, 1946) was ground with 2·5 ml. of the upper (aqueous) phase from solvent *A* and a slurry was then made with 40 ml. of the

lower layer. The slurry was pipetted into a siliconed chromatography tube, 25 cm. long and 1·3 cm. int. diam., in five portions, each section being packed under pressure, and the walls of the tube were washed with solvent after each addition. The pressure was maintained for a few seconds after the liquid level had cleared the surface of the column, but care was taken not to let the surface crack. The column should be 9·3–9·5 cm. high and it should have a firm level surface. 8-Hydroxyquinoline (25 mg. dissolved in 1 ml. of solvent *A*) was then added to the column and washed through with fresh solvent. This removed metal ions and was also a visible test of the adequacy of packing. The column was then clamped over a drop-counting fraction collector.

Fractionation on the column. A solution containing the β-amino alcohol hydrochlorides was run into an evaporating basin and taken to dryness in a vacuum desiccator. The residue was then dissolved in 1·5 ml. of solvent *A* and transferred to the column. After the solvent had drained into the column the evaporating dish and sides of the chromatography tube were washed five times with approx. 1 ml. lots of the solvent. A volume (6 ml.) of the solvent was then pipetted on to the column and the chromatography tube was attached by a rubber stopper to a glass-capillary tube that led to a 430 ml. mixing flask containing a glass-covered stirring bar mounted over a magnetic stirrer. The inlet to the mixing flask was a 500 ml. separating funnel having a capillary stem. Solvent *A* (400 ml.) was added to the mixing flask and about 20 ml. to the separating funnel. Both inlet and outlet tubes should be well under the surface of the solvent. Pressure was then gradually applied from a cylinder of nitrogen fitted with a reducing valve and outlet-pressure gauge, until the rate of flow was about 8 ml./hr. The top of the separating funnel was then closed and the cylinder valve shut without further adjustment of the reducing valve. The remaining solvent in the separating funnel was then removed and the funnel was filled with solvent *B* to which had been added 15 ml. of ethanol/100 ml. of solvent. The gas cylinder was again connected and the valve opened. After waiting for the pressure to build up, the separating-funnel tap was opened and fractions of 2·6 ml. were collected in 13 cm. × 1·6 cm. test tubes. All the above operations were carried out in a room controlled at 27·5±1°.

The fractions, in racks of 25 tubes, were evaporated almost to dryness in a water bath after the addition of 1 drop of 5% (w/v) phosphoric acid in ethanol and a small glass bead. The temperature of the bath was raised slowly so as to eliminate the danger of loss by ebullition. The tubes were finally dried for 30 min. *in vacuo* over P_2O_5 and KOH.

Comments. When solvents *A* and *B* are mixed the buffer solution tends to precipitate. The addition of ethanol minimizes the effect and the small amount of aqueous phase that separates in the mixing flask during the first few hours of the chromatographic run is gradually re-adsorbed and does not interfere with the fractionation.

The air locks formed between the solvent and stoppers in the mixing flask and chromatographic tube are necessary to prevent contact of the solvent with the rubber. A mixing chamber of the type described by Moore & Stein (1954) could not be used in the present work because the plasticizer which is a component of the synthetic (polyvinyl chloride) tubing was extracted in the solvents and caused interference.

Analysis of fractions. β-Amino alcohols react with ninhydrin to give the familiar blue colour, but the colour yield depends on the amino alcohol concerned and is always lower, sometimes much lower, than that given by the corresponding amino acid; ethanolamine, for instance, gives 70% and alaninol 16% of the colour yield obtained from leucine. β-Amino alcohols also react with periodate to give formaldehyde, and all those that we are concerned with give 1 mol. equivalent except ethanolamine and serinol, which give 2. A simple colorimetric method of estimating formaldehyde, based on the specific reagent (chromotropic acid) of Eegriwe (1937), has been developed, and has been used in the present research as an indirect method for the quantitative estimation of β-amino alcohols.

Estimation of β-amino alcohols as formaldehyde after periodate oxidation

Reagents. Saturated solutions of K_2HPO_4 and $Na_2S_2O_5$. Periodate reagent: $NaIO_4$ (1·07 g.) was dissolved in water (44·7 ml.) and 5·3 ml. of 2N-NaOH added. Chromotropic acid: the acid (0·9 g.; Judex special reagent) and $Na_2S_2O_5$ solution (0·5 ml.) were adjusted to 25 ml.; the solution was stored in a dark bottle. H_2SO_4: 98–100%, N-free. All reagents are stable over several weeks.

Procedure. To each tube in the rack of 25 was added 0·1 ml. of the K_2HPO_4 solution followed by 0·8 ml. of water and, after mixing, 1 drop (approx. 0·02 ml.) of periodate solution. The oxidation was allowed to proceed for 4 min., when 1 drop of $Na_2S_2O_5$ solution was added to destroy the excess of periodate. Chromotropic acid solution (0·1 ml.) was then pipetted into each tube and, after cooling in an ice bath, 4 ml. of H_2SO_4 was slowly added from a burette. The tubes were shaken and the colour was developed by heating in a boiling-water bath for 10 min. The tubes were again cooled, diluted with 5 ml. of water and the optical density determined at 570 mμ or in a photoelectric colorimeter fitted with a suitable filter.

Values for each peak were obtained from a standard curve (Fig. 1) plotted from readings obtained with a series

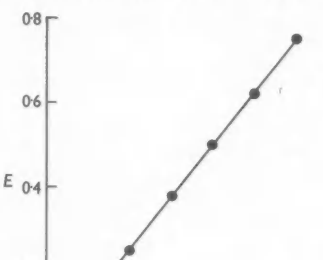

Fig. 1. Typical standard curve from formaldehyde given on periodate oxidation of serine. Sulphuric acid free from nitrogen was used.

of solutions of serine of known concentration or a standardized formaldehyde solution. The standard curve should be checked at frequent intervals and blank readings for the reagents should be of the order of 0·02 on the optical-density scale.

Comments. The colour developed from formaldehyde is stable for 24 hr. provided that the tubes are screened from direct sunlight. Care must be taken to check the standard curve whenever a fresh lot of H_2SO_4 is used, as on two occasions gross interference has been observed. In each case the colour yield was approximately half the maximum given by other lots of H_2SO_4, though it was still proportional to the amount of formaldehyde present. Russell (1944) has reported a similar anomaly with the colorimetric determination of acetaldehyde by *p*-hydroxydiphenol in the presence of H_2SO_4, which she traced to the presence of traces of nitrates or nitrites in the particular sample of H_2SO_4 used.

Identification of β-amino alcohols

Paper chromatography. To check the identity of the amino alcohol peaks the fractions concerned are collected and the solvent is removed. Water (1 ml.) is added to each fraction and 0·5 ml. taken for the estimation of formaldehyde given on periodate oxidation. The remainders of the fractions from each peak are then bulked and, after concentration to 1–2 ml., passed through a column (1 cm. × 5 cm.) of Dowex-2 × 10 in the free-base form. The eluate is then acidified with HCl, evaporated to dryness and the β-amino alcohol hydrochloride subjected to paper chromatography with either propanol–water (80:20, v/v) or the solvents described by Fromageot, Jutisz, Meyer & Penasse (1950), in the manner described below.

When dealing with products derived from esterified and reduced proteins or obtained from the buffered columns the presence of inorganic salts can interfere with the paper chromatography. In such cases the following procedure was found useful. In phenol (70 g.)–water (30 ml.) to which had been added 5% (v/v) aq. NH_3 soln. (sp.gr. 0·880), the amino alcohols travelled with the solvent front and the clearly visible salt spot was some distance behind. A sample of material from the amino alcohol peak, and several suitable marker spots, were placed on a line 1·5 cm. from the edge of a sheet of Whatman no. 1 filter paper and run as an ascending chromatogram for 6 cm. with the phenol solvent just mentioned. After removal of the phenol by drying at 45° and washing the paper with dry ether, the section containing the salt was cut off and the remainder of the paper chromatographed in the usual way with propanol–water or the other solvent mixtures mentioned above. The colour development with ninhydrin is slower than with α-amino acids and in view of the differences in intensity of colour given by different β-amino alcohols, to which attention has already been drawn, a visual comparison of spots can be very misleading.

Oxidation to α-amino acids. In most cases this can be effected smoothly with Ag_2O and the procedure can be useful. The amino alcohol fraction containing from 1 to 10 μmoles (10 ml.) was run into a glass tube and Ag_2O (400 mg.) added. The tube was then sealed and heated overnight at 100°, the contents being shaken occasionally during the first 2 hr. After cooling, the tube was opened, HCl (0·4 ml.) added and the contents of the tube heated in a boiling-water bath for 5 min. to precipitate AgCl. After centrifuging and washing twice with water the combined liquors were evaporated to dryness *in vacuo* at 40° and the residue was taken up in water. The amino acids present were then separated from any unoxidized material and salts by adsorption on a column (1 cm. × 5 cm.) of Dowex-2 × 10 in the free-base form and washing the column with water until it was salt-free. The amino acids were then eluted from the column with N-HCl (20 ml.) and, after removal of excess of mineral acid by evaporation, they were identified by paper chromatography. Ethanolamine and leucinol gave the corresponding amino acids in 75% yield, but, as one might expect, the recoveries of tyrosine from tyrosinol were low and erratic.

RESULTS

A typical effluent concentration curve from a mixture of the β-amino alcohols listed is given in Fig. 2 (*a*), and the recoveries are summarized in Table 1. The effluent volume for a given amino alcohol was found to be reproducible to ± 5% and the position of the peaks relative to one another varied by no more than ± 2%. Rates of flow between 4 and 12 ml./hr. appeared to have no effect on the shape of the peaks but increasing the temperature over the range 18–30° gave progressively sharper peaks.

Fig. 2 (*b*) shows the concentration curves obtained from the β-amino alcohol hydrochloride fractions of hydrolysates of 16 mg. of reduced insulin and reduced insulin ester that had been separated from the amino acids by the method described in the first paper of this series (Chibnall & Rees, 1958). Before chromatography most of the lithium was removed from the β-amino alcohol hydrochloride fraction by passing it through a column (20 cm. × 1·5 cm.) of Dowex-2 × 10 in the free-base form. The amino alcohol hydrochloride fraction, in a volume of 1–2 ml., was added to the column and eluted with CO_2-free water. The eluate was collected in fractions of 1·3 ml. and the lithium was located either by a flame test or by spot-testing the fractions on Universal Indicator paper. The lithium came through as a narrow band in fractions 9 and 10, and the β-amino alcohols as

Table 1. *Recoveries of β-amino alcohols from mixtures chromatographed on buffered silica gel*

β-Amino alcohol	β-Amino alcohol (μg.) Taken	Found	Recovery (%)
Phenylalaninol	147	144	98
Leucinol / Isoleucinol	141	136	96
Valinol	90·4	88·6	98
Tyrosinol	171	165	96
Propanolamine	153	150	98
Aspartidiol	142	138	97
Ethanolamine	62·7	61·1	99
Mixture	907·1	882·7	97·4

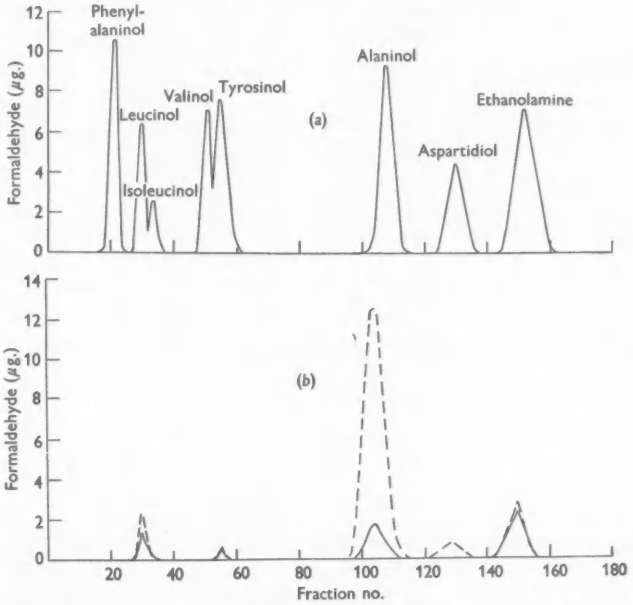

Fig. 2. (*a*) Chromatographic separation of β-amino alcohols from a synthetic mixture on a column of buffered silica gel (1·3 cm. × 9·5 cm.), gradient elution with ethanol–chloroform being used. (*b*) Separation of β-amino alcohols from hydrolysates of insulin ester hydrochloride reduced with $LiBH_4$ (broken line) and insulin reduced with $LiBH_4$.

one diffuse peak extending from fractions 9 to 46. Fractions 11 to 46 were bulked and, after the addition of 2–3 drops of conc. HCl, taken to dryness for chromatography. The addition of acid to solutions of β-amino alcohols before concentration is necessary to prevent losses due to volatility. Periodate formaldehyde estimations were carried out on portions from fractions 5 to 10 and the final results were corrected for the amount of β-amino alcohols in fractions 9 and 10, usually of the order of 2–3 %.

DISCUSSION

The conditions used in the column-chromatographic procedure outlined above were chosen to give an adequate separation, in the minimum number of fractions, of the β-amino alcohols obtained from certain proteins that were being investigated primarily for other purposes. Under such conditions, and with a more complex mixture of β-amino alcohols than that portrayed in Fig. 1, some overlapping occurred. Glutamidiol, for instance, was located between aspartidiol and ethanolamine and was not completely separated from them, and serinol occupied a similar position. By making use of a larger column, by increasing the capacity of the mixing flask, by altering the pH of the buffer solution or by a combination of these changes it should be possible to obtain a separation of the components of a mixture different from that found in the present work.

From preliminary experiments with a buffered column of Celite it is clear that adsorption as well as partition was taking part in the separation with silica gel. When using a column (1·3 cm. × 17 cm.) of buffered Celite (8 g.) packed by the method of Chibnall & Spahr (1958) a separation similar to that shown in Fig. 2 was obtained. Nevertheless, under the conditions used, no separation was obtained between leucinol and isoleucinol, and tyrosinol and valinol overlapped to give an asymmetrical peak.

SUMMARY

1. The quantitative chromatographic separation of β-amino alcohols on partition columns of buffered silica gel, with ethanol–chloroform mixtures as eluting agents, is described. The recoveries vary between 96 and 100 %.

2. A method for the quantitative estimation of β-amino alcohols, based on the formaldehyde given on periodate oxidation, has been developed.

3. The procedure has been used to separate and identify the β-amino alcohols present in the hydrolysates of esterified and reduced proteins.

4. The use of silver oxide to oxidize β-amino alcohols to the corresponding α-amino acid is described.

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Studies on the Amide and *C*-Terminal Residues in Proteins

5. ESTIMATION OF ASPARAGINE AND GLUTAMINE RESIDUES

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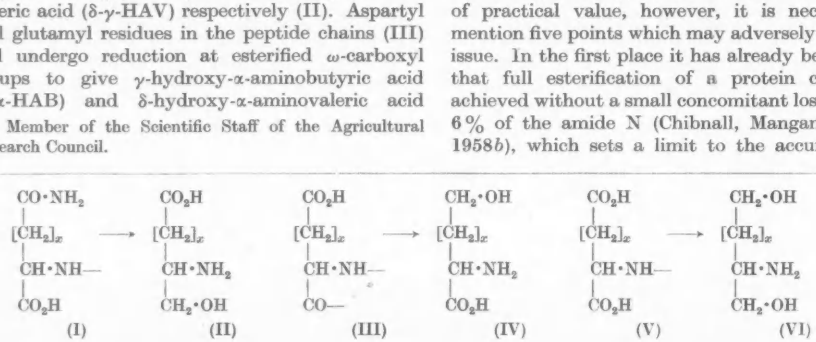
In the introduction to the first paper of this series (Chibnall & Rees, 1958) an outline was given of the modifications that an esterified protein could undergo on reduction with lithium borohydride, and how the various changes produced would become manifest after hydrolysis. In the present paper we deal with one particular aspect of this phenomenon, which offered the possibility of estimating the total number of asparagine and of glutamine residues in the protein molecule concerned. Subject to certain qualifications to be discussed later, asparaginyl and glutaminyl residues which occur in the peptide chains will remain intact until hydrolysis, when they will be converted into aspartic and glutamic acids respectively, whereas those occupying *C*-terminal positions (I) will undergo reduction at the esterified α-carboxyl group to give, eventually, γ-hydroxy-β-aminobutyric acid (γ-β-HAB) and δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV) respectively (II). Aspartyl and glutamyl residues in the peptide chains (III) will undergo reduction at esterified ω-carboxyl groups to give γ-hydroxy-α-aminobutyric acid (γ-α-HAB) and δ-hydroxy-α-aminovaleric acid (δ-α-HAV) respectively (IV), whereas if they occupy *C*-terminal positions (V) they will give the corresponding diols (VI). Products (II) and (VI), having hydroxyl and amino groups attached to adjacent carbon atoms, are susceptible to periodate oxidation, which makes it possible to devise a procedure for determining the various residue species in the protein concerned.

Aspartic and glutamic acids (estimated with ninhydrin), as well as δ-γ-HAV and γ-β-HAB (estimated as periodate-formaldehyde), are separated on a column of Dowex-50 by elution with hydrochloric acid. There is a partial overlap between δ-α-HAV and γ-β-HAB so that the former cannot be estimated by ninhydrin. δ-α-HAV and γ-α-HAB have been computed by difference. The two diols (VI) are estimated after separation on a column of silica gel (Rees, 1958).

Before presenting evidence that such a scheme is of practical value, however, it is necessary to mention five points which may adversely affect the issue. In the first place it has already been shown that full esterification of a protein cannot be achieved without a small concomitant loss of about 6% of the amide N (Chibnall, Mangan & Rees, 1958*b*), which sets a limit to the accuracy with



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which the amide distribution can be determined with proteins of high molecular weight, as we shall see later for β-lactoglobulin. Secondly, reductive cleavage of peptide bonds (Crawhall & Elliott, 1955) may occur, so that residues in peptide chains will undergo modification as though they were present in the *C*-terminal position. Aspartyl and glutamyl residues (III) therefore will be found in the reduced ester hydrolysate as diols (VI), and the corresponding amide residues as the periodate-labile hydroxyamino acids (II). As will be shown later, these changes can be quantitatively determined and allowed for. Thirdly, Crawhall & Elliott (1955) have suggested the possibility that lithium borohydride might reduce the acid amide group. Evidence presented later shows that this may occur, but to a negligible extent. Fourthly, the possible occurrence of β-aspartyl and γ-glutamyl peptide linkages in the chains must be considered. Their presence would lead to the occurrence of more of the respective hydroxyamino acid (II) in the hydrolysate of the reduced ester than in that of the reduced protein, and confusion with *C*-terminal asparagine or glutamine residues would be possible. Lastly, there is the possible occurrence of a terminal amide residue, other than that of asparagine or glutamine. This would be indicated by an unbalance in the reduced ester between the number of amide groups on the one hand and the total number of aspartic acid, glutamic acid and hydroxyamino acid (II) residues on the other. The evaluation would thus be susceptible to the overall errors of analysis and would become progressively less valid with increase in molecular weight of the protein concerned.

EXPERIMENTAL

Materials

L-Pyrrolidonecarboxylic acid. This was prepared from L-glutamic acid by the method of Lichtenstein (1942). The product gave no colour with ninhydrin.

δ-Hydroxy-γ-aminovaleric acid. We have to thank Professor Linderstrøm-Lang for a sample prepared by Sørensen (1905).

γ-Hydroxy-β-aminobutyric acid. This was prepared from α-aminobutyrolactone by the method of Livak, Britton, Vander Weele & Murray (1945).

α-γ-Diaminobutyric acid. We have to thank Dr L. C. Craig for a sample of this compound.

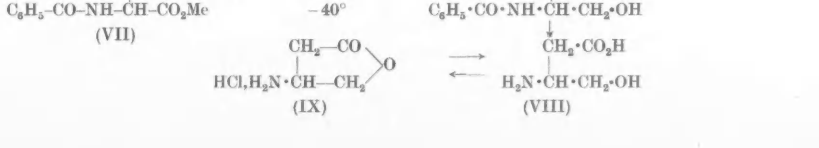
Proteins. The samples have been described elsewhere (Chibnall, Mangan & Rees, 1958*a*).

Synthesis of γ-hydroxy-β-aminobutyric acid (γ-β-HAB)

The following synthesis was carried out in 1951 before the technique for handling $LiBH_4$ had been fully explored. *N*-Benzoylasparagine methyl ester (VII) was reduced with $LiAlH_4$ under mild conditions. The amide group was largely unaffected, and after hydrolysis the required hydroxyamino acid (VIII) was isolated and purified as β-aminobutyrolactone hydrochloride (IX).

N-Benzoylasparagine methyl ester. *N*-Benzoylasparagine (10 g., Pauly & Weir, 1910), somewhat contaminated with the free acid owing to hydrolysis of the amide group during benzoylation, was dried *in vacuo* and suspended in methanolic 0·1 N-HCl. The mixture was shaken at 25° for 24 hr., the product going into solution as the esterification proceeded. The solvent was removed by evaporation *in vacuo*, and the residue, after standing for some hours in a vacuum desiccator over NaOH, was ground to a fine powder and extracted exhaustively with warm ligroin. The final residue was taken up in warm acetone, from which it crystallized on cooling. Yield of *N*-benzoylasparagine methyl ester, 6·7 g. (63%). The ligroin liquors were collected, reduced in volume and cooled, when *N*-benzoylaspartic acid dimethyl ester separated in needles (2·83 g.).

β-Aminobutyrolactone hydrochloride. *N*-Benzoylasparagine methyl ester (1·1 g.) was placed in a reaction vessel fitted with a stirrer and tetrahydrofuran (10 ml.) added. The mixture was then cooled to −40° and 1·6 M-$LiAlH_4$ (14 ml.) added slowly with stirring. After 30 min. the cooling bath was removed and the reaction temperature allowed to rise slowly to room temperature during 30 min. The mixture was then cooled again to −40°, ether saturated with water was added and then a slight excess of ethanolic HCl. The solvents were removed by evaporation, and the residue was hydrolysed with 5 N-HCl (25 ml.) for 4 hr. After removing excess of mineral acid the residue was dissolved in water, and neutralized with $Ba(OH)_2$ to precipitate $Al(OH)_3$, which was centrifuged off and washed. The clarified liquors were collected, made just alkaline with $Ba(OH)_2$ and evaporated *in vacuo* to remove ammonia. Barium was then removed as $BaSO_4$, the solution was reduced to a small volume, transferred to a column (3·5 cm. × 25 cm.) of Dowex-50, and eluted with N-HCl. Fractions (5 ml.) were collected, and from each a portion (0·05 ml.) was removed for flame test (Li) or periodate analysis. Lithium was eluted between fractions 60 and 80, and periodate-reducing material between fractions 97 and 130. The latter fractions were combined and reduced to a syrup *in vacuo*. The γ-lactone hydrochloride crystallized from a small volume of hot ethanol; yield, 263 mg. (45%), m.p. 185–186° (Found: C, 35·23; H, 5·78; N, 10·19. $C_4H_7O_2N,HCl$ requires C, 34·91; H, 5·86; N, 10·18%).



The γ-lactone hydrochloride is very stable, and gives no formaldehyde under the usual conditions of periodate oxidation (Rees, 1958). Treatment with a phosphate buffer (pH 11) at 100° for 5 min. opens the ring and a 75% recovery of formaldehyde is obtained. Boiling under reflux with 5N-HCl produces an equilibrium mixture containing about 50% of the free acid; a similar mixture results when either the lactone or free acid is eluted from a column of Dowex-50 with N-HCl. Paper chromatography with butanol–acetic acid–water (4:1:5, by vol.) does not open the ring, and on spraying with ninhydrin a yellow spot is obtained, $R_F = 0{\cdot}22$.

γ-Hydroxy-β-aminobutyric acid. The method used was that of Fischer & Blumenthal (1907) for opening the ring of α-aminobutyrolactone hydrobromide. The lactone hydrochloride (97 mg.) was dissolved in water and shaken with freshly precipitated and washed Ag_2CO_3. The AgCl and excess of carbonate were centrifuged off and the supernatant and washings treated with H_2S to remove Ag. More conveniently, the combined HCl can be removed on a column of Amberlite IR-4B. The lactone solution was evaporated on an open water bath, taken up in water and the operation repeated several times. On reducing to a syrup the free acid crystallized in large prisms; yield, 55 mg. (55%). It was recrystallized from water; m.p. 232–233·5° (decomp.); periodate–formaldehyde 97% of theoretical (Found: C, 40·31; H, 7·47; N, 11·56. $C_4H_9O_3N$ requires C, 40·33; H, 7·62; N, 11·76%). On paper chromatography the acid ran as one spot and gave a normal ninhydrin colour; $R_F = 0{\cdot}31$ in butanol–acetic acid–water (4:1:5, by vol.). Jollès & Fromageot (1951) claim to have prepared the acid by reduction of *N*-benzyloxycarbonyl-DL-asparagyl-β-ethylglycinate α-benzyl ester with $LiAlH_4$, followed by hydrolysis. The crude mixture was separated by paper chromatography. The few milligrams of material obtained were shown to be oxidized by periodate and to give a discrete spot on paper chromatography but were not otherwise characterized.

Synthesis of δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV)

Jollès & Fromageot (1952) claim to have prepared this hydroxyamino acid by reduction of the α-benzyl ester of ethyl *N*-benzyloxycarbonylglutamyl-γ-glycinate with $LiAlH_4$, but the few milligrams of material obtained were not satisfactorily characterized. In the present case the methyl ester of pyrrolidonecarboxylic acid (X) was reduced with $LiAlH_4$ to pyrrolidonyl alcohol (XI), the ring of which opened on hydrolysis to give δ-hydroxy-γ-aminovaleric acid (XII).

L-Pyrrolidonecarboxylic acid (2·2 g.) in 85% ethanol, containing 1·5 ml. of N-HCl, was treated at 0° with diazomethane (1·1 g.) in ether. On removing the solvent *in vacuo* the ester was obtained as a colourless oil which was not further purified. It was reduced with $LiAlH_4$ under the conditions described above, and, after hydrolysis, was separated on a (3·5 cm. × 25 cm.) column of Dowex-50 (fractions 78–120), N-HCl being used for elution. On evaporation δ-γ-HAV hydrochloride was obtained as an oil which crystallized in needles on standing for several months. After treatment on a column of Amberlite IR-4B the free acid was evaporated *in vacuo* and an equal volume of alcohol added. The precipitated material dissolved on warming, and on cooling to 0° it crystallized in clumps of needles, m.p. 160–161·5°; periodate–formaldehyde, 98·5–100% theory; (Found: C, 45·3; H, 8·32; N, 10·45. $C_5H_{11}O_2N$ requires C, 45·1; H, 8·33; N, 10·52%). On paper chromatography it gave a normal ninhydrin colour; $R_F = 0{\cdot}21$ in butanol–acetic acid–water (4:1:5, by vol.).

Methods of estimation

Amide nitrogen. This was estimated as described by Chibnall *et al.* 1958*b*.

Hydrolysis of proteins. This was done as described by Chibnall & Rees (1958).

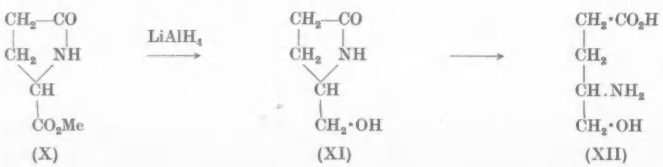
Ornithine and α-γ-diaminobutyric acid. These were estimated by the gradient-elution procedure of Moore & Stein (1954). They emerge together, following ammonia and in front of lysine. As ornithine alone gives a colour by the specific method of Chinard (1952) the two bases can be separately estimated by treating portions of the relevant fractions by this method as well as by ninhydrin. Amino alcohols emerge in this region; if present they can be detected by periodate oxidation, but the quantitative estimation as formaldehyde is unreliable because of the presence of thiodiglycol and BRIJ 35 (polyoxyethylene lauryl alcohol) in the elution liquors.

L-*Glutamic acid.* This was estimated by the decarboxylase method of Gale (1945). The bulk supply of *Clostridium welchii*, S.R. 12, was prepared by the procedure of Krebs (1950). After hydrolysis by the usual procedure the recovery of the acid was 96±1%; a correction factor of 4% has accordingly been applied. In agreement with both these workers we find that the enzyme preparation is without action on L-aspartic acid (cf. Meister, Sober & Tice, 1951).

L-*Aspartic acid.* The shortened procedure of Krebs (1950) was used so as to avoid possible interference from cysteic acid. In agreement with Krebs (1953) it was found that special care must be taken to ensure quantitative liberation of CO_2 by adjusting the reaction mixture to below pH 5. The recovery was 95±1% and the correction factor 5%.

Procedures

Unless otherwise stated the protein esters were prepared with methanolic 0·1N-HCl at 25° for 24 hr. and were reduced with 0·3M-$LiBH_4$ (eightfold molar excess) in tetrahydrofuran under reflux for 6 hr. (Chibnall & Rees, 1958).



(X) (XI) (XII)

Column analysis for aspartic acid, glutamic acid, γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid. To avoid buffered systems which would interfere with the oxidation of the hydroxyamino acids by periodate, Dowex-50 was used in the hydrogen form and the amino acids were eluted with HCl (Stein & Moore, 1949*a*). The column (50 cm. × 1 cm.) was water-jacketed to maintain 18–19° and packed in 4 cm. zones. It was first washed with N-NaOH until the eluate was alkaline, and a further 50 ml. run through. Water was then applied until the eluate was neutral. The column was regenerated with N-HCl, which was run through until the flame test for Na^+ ion was negative. The sample to be chromatographed (2·2–2·5 mg. of N) in N-HCl was then added and washed into the column with 4 × 1 ml. of N-HCl. The usual elution procedure with N-HCl was then applied and 140 fractions (1 ml.) were collected. From each of these sample (0·2 ml.) was withdrawn, neutralized with N-NaOH (0·2 ml.) and treated with ninhydrin (Chibnall *et al.* 1958*a*). If the temperature control had been adequate a good separation was obtained (Fig. 1). From fraction 141 onwards the fraction size was increased to 2 ml. and the tubes, in racks of 25, were dried overnight in a vacuum desiccator (0·5 mm.) over P_2O_5 and NaOH. The residual material was dissolved in phosphate buffer (pH 11, 0·1 ml.) and heated at 100° for 5 min. to open the γ-lactone ring, a treatment that does not affect the estimation of δ-γ-HAV. The periodate–formaldehyde procedure of Rees (1958) was then applied.

Recovery of hydroxyamino acids. After hydrolysis for 24 hr. with HCl–formic acid and treatment under the usual conditions used for proteins the recovery of δ-γ-HAV, estimated by periodate oxidation, was 85%. Attention has already been drawn to the equilibrium established between γ-β-HAB and its lactone on acid hydrolysis, during column fractionation and on periodate analysis of the column effluents. To allow for these various incidental interferences, and also for destruction during hydrolysis, it was necessary to work under standard conditions. Accordingly, insulin with 2% of γ-β-HAB and β-lactoglobulin with 1% of δ-γ-HAV were passed through the hydrolysis and column treatment. The recovery of the former acid was 63·8% and of the latter 72%; corresponding correction factors have been applied in all the analyses quoted below. These low recoveries are due not to extensive destruction on hydrolysis or to incidental losses but to ring formation under the conditions used (cf. Chibnall, Rees & Richards, 1958). The recovery of aspartic and glutamic acids after hydrolysis and treatment on the column was 100±2% and no correction factors have been applied.

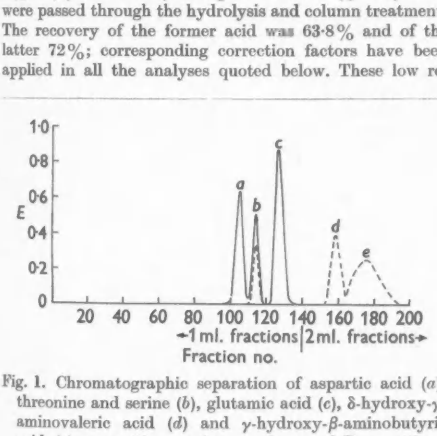


Fig. 1. Chromatographic separation of aspartic acid (*a*), threonine and serine (*b*), glutamic acid (*c*), δ-hydroxy-γ-aminovaleric acid (*d*) and γ-hydroxy-β-aminobutyric acid (*e*) on a 50 cm. × 1 cm. column of Dowex-50 × 8 eluted with N-HCl (——, estimated by ninhydrin; - - - -, estimated by formaldehyde given on periodate oxidation).

Comments. The procedure gives a sharp separation of the four products required. If the temperature of the column is allowed to rise above 20° the serine–threonine peak tends to move forward and the separation from aspartic acid may be impaired. If portions of fractions 105–115 are developed with ninhydrin to give the serine–threonine peak and a similar series of portions with periodate to give a serine peak, a reasonably accurate value for threonine can be obtained by difference. When the elution is carried through to the fourth day the amino alcohols emerge, but because of the time lag the order of emergence and the recoveries have not been investigated in detail. δ-α-HAV and γ-α-HAB emerge just behind δ-γ-HAV and γ-β-HAB respectively, but they must be developed with ninhydrin and reasonable resolution of δ-α-HAV from γ-β-HAB has not yet been achieved. This might be possible on a longer column, or at a higher pH at which the expected differences in the p*K* values of the carboxyl groups would be effective.

RESULTS AND DISCUSSION

Loss of amide nitrogen on reduction. A direct reduction of the amide group in residues of glutamine and asparagine would lead to the presence of ornithine and α-γ-diaminobutyric acid respectively in the hydrolysate of reduced protein or protein ester. When search was made for these two basic amino acids the amounts found were so small that it was necessary to charge the Moore & Stein (1954) column with four times the normal load of hydrolysate. A reduced insulin ester gave 0·1 mole of ornithine/mole of protein (5732 g.) and a reduced β-lactoglobulin ester 0·23 mole/mole of protein (37 000 g.). In neither case was α-γ-diaminobutyric acid detected.

Reductive cleavage of the amide bond would also lead to a loss of amide N. According to our experience, however, the observed loss is no more than that which should accompany the direct reduction mentioned above. For insulin, the protein (dispersed as described by Chibnall & Rees, 1958) showed a fall of 0·04 and the ester 0·1 mole/mole of protein (5732 g.) respectively. The ester and reduced ester of β-lactoglobulin gave the same value within experimental limits, as one would expect if the actual loss were no greater than 0·23 out of 26·2 moles/mole of protein (37 000 g.). It can be assumed therefore that, within the limits claimed for the present procedure, the loss of amide N on reduction with $LiBH_4$, about 1·0–1·5% of the total amide N, is negligible. The recent work of Davis (1956) led to a similar conclusion.

Analysis of β-lactoglobulin

Protein dicarboxylic acids. The values for glutamic acid obtained by the decarboxylase procedure, which is optically specific, and by column analysis, which is not specific, are in agreement with each other (Table 1) and in slight excess (2 residues/mole) over those obtained with a starch column (Stein & Moore, 1949*b*) and by isotope dilution (Foster, 1945). The aspartic acid values are in agreement throughout. It is clear from these results, for which an accuracy closer than 1 residue/mole cannot be claimed, that the protein hydrolysate does not contain any appreciable proportion of racemized glutamic acid. This finding is of interest in connexion with the work of Bailey, Chibnall, Rees & Williams (1943), who claimed to have isolated, in terms of residues/mole, 49 of L- and 5 of D-glutamic acid as the hydrochloride, and only 28 of L-aspartic acid as the Cu salt. It would appear that when aspartic acid is present in fairly high concentration, as it would be in this particular hydrolysate, it can be salted out as the hydrochloride along with that of L-glutamic acid, and the assumed presence of D-glutamic acid must be ascribed at least in part to this occurrence, for aspartic acid hydrochloride has a lower positive rotation than glutamic acid hydrochloride. We have, indeed, recently tested by paper chromatography some of the (assumed) partially racemized glutamic acid–hydrochloride fractions isolated by Bailey and his colleagues in the above-mentioned work, and they contain aspartic acid, but in amount too small to be detectable by methods available in 1943.

Reduced protein. Reductive cleavage of peptide bonds has occurred to a small extent and the hydroxyamino acids thereby produced could have come from corresponding residues of either the free dicarboxylic acids or the acid amides. The evidence given later shows that the amide residues alone are concerned.

Reduced protein ester. Reductive cleavage of peptide bonds to the same small extent was again encountered. In this case, however, the γ-β-HAB and the δ-γ-HAV must have had their origins in asparaginyl and glutaminyl residues respectively, as aspartyl and glutamyl residues would have given rise to the corresponding diols, the presence of which was not observed (Chibnall & Rees, 1958). The hydroxyamino acids have been accordingly included in the summation of the aspartic and glutamic acid residues given in Table 1, which are collectively in good agreement with the amide N of the ester concerned. The amide N lost during esterification (two groups) may have come exclusively, as with insulin, from asparagine residues, but we have preferred, arbitrarily, to assume that the loss is distributed equally between the two amide species (Table 3).

Analysis of lysozyme

The aspartic and glutamic acid analysis of the protein (Table 2) is in agreement with that of Thompson (1955). When the protein is esterified with methanolic 0·1 N-HCl at 25° for 24 hr. there is a fall in the amide N (Chibnall *et al.* 1958*a*) from 17·9 to 17·0 groups/mole. The data for the reduced protein (Table 2) leave no doubt that the distribution of residues set out in Table 3 is correct. Smith, Kimmel & Brown (1954) deduced from the electrophoretic mobility studies of Alderton, Ward & Fevold (1945) that the free carboxyl groups in this protein were predominantly aspartyl groups.

Table 1. *Analysis of β-lactoglobulin*

Protein samples A and B, see Chibnall *et al.* (1958*a*). Treatment: H, hydrolysed; R, reduced and hydrolysed. Methods of analysis: E, enzyme; C, column of Dowex-50. Results are expressed as residues/mole (37 000 g.).

Material	Treatment	Method of analysis	1 Aspartic acid	2 γ-β-HAB	3 1+2	4 Glutamic acid	5 δ-γ-HAV	6 4+5	7 3+6	Amide N
Protein A	H	E	30·1	—	—	49·9	—	—	—	—
Protein A	H	C	31·7	—	—	50·5	—	—	—	—
Protein B	H	E	31·1	—	—	49·5	—	—	—	—
		Mean	31·0	—	31·0	50·0	—	50·0	81·0	28·0
Protein A	R	E	29·8	—	—	—	—	—	—	—
Protein B	R	E	30·4	—	—	49·6	—	—	—	—
Protein B	R	C	30·4	0·8	—	49·0	1·7	—	—	—
		Mean	30·2	0·8	31·0	49·3	1·7	51·0	82·0	—
Ester A	R	E	8·6	—	—	15·8	—	—	—	—
Ester A	R	C	8·2	—	—	15·0	—	—	—	—
Ester B	R	E	8·4	—	—	15·5	—	—	—	—
Ester B	R	C	8·0	0·9	—	15·8	1·5	—	—	—
		Mean	8·3	0·9	9·2	15·5	1·5	17·0	26·2	26·0

Table 2. *Analysis of lysozyme, β_1-lactoglobulin and ox insulin*

Treatment: H, hydrolysed; R, reduced and hydrolysed. Results are expressed as residues/mole (lysozyme, 14 700 g.; β_1-lactoglobulin, 37 000 g.; insulin, 5732 g.).

Material	Treatment	1 Aspartic acid	2 γ-β-HAB	3 1+2	4 Glutamic acid	5 δ-γ-HAV	6 4+5	7 3+6	8 Amide N
β_1-Lactoglobulin	H	30·6	—	30·6	49·8	—	49·8	80·4	27·7
β_1-Lactoglobulin	R	30·0	0·8	30·8	49·0	1·4	50·4	81·2	—
β_1-Lactoglobulin ester	R	8·2	0·9	9·1	15·6	1·33	16·9	26·0	25·9
Lysozyme	H	20·1	—	20·1	4·95	—	4·95	25·05	17·9
Lysozyme	R	18·4	0·67	19·07	3·87	1·0	4·87	23·94	—
Lysozyme ester	R	11·3	0·86	12·16	3·64	1·03	4·67	16·83	17·0
Insulin	H	2·94	—	2·94	6·90	—	6·90	9·84	5·82
Insulin	R	2·75	0·08	2·83	6·72	0·17	6·89	9·72	5·78
Insulin ester	R	1·6	0·89	2·49	3·0	0·18	3·18	5·67	5·45

Table 3. *Number of asparagine and glutamine residues in different proteins*

Results are expressed as residues/mole of protein, allowance being made for amide loss during esterification.

Residue	β-Lactoglobulin (mol.wt. 37 000)	β_1-Lactoglobulin (mol.wt. 37 000)	Lysozyme (mol.wt. 14 700)	Ox insulin (mol.wt. 5732)
Asparaginyl	10	10	13	3
Glutaminyl	18	18	5	3
Aspartyl	21	21	7	—
Glutamyl	32	32	—	4

Ohno (1954) claimed to have shown by hydrazinolysis that the protein contains 1 aspartyl, 12 asparaginyl and 4 glutaminyl residues/mole.

Analysis of β_1-lactoglobulin

The mol.wt. of this protein has not been determined, but the amide N would allow 28 groups/mole of 37 000 g., the same as β-lactoglobulin. The data given in Tables 2 and 3 fail to reveal any difference between the two proteins.

Analysis of insulin

This protein is of particular interest in the present studies. In the first place, its amino acid structure is known, so that deductions based on the procedure under review can be verified. Secondly, the sample of insulin used in these studies, which was a commercial preparation that had been specially purified for research purposes, has been shown to be deficient in amide N (5·82 instead of the expected 6 groups/mole, Chibnall *et al.* 1958*a*). The data collected in Table 2 show that reductive cleavage of peptide bonds has occurred, and as the amount of δ-γ-HAV produced from the ester was the same as that from the protein itself, it may be inferred, as discussed above, that glutaminyl and not glutamyl residues were concerned. The computed number of glutamine residues in the reduced ester (Table 2, column 6) is, indeed, in slight excess of the number (3/mole) required to satisfy the known structure and reflects, according to our experience, the difficulty of fully esterifying the glutamyl residues in this particular protein. The γ-β-HAB given by the reduced protein may have been derived from asparaginyl residues in the peptide chains, for insulin does not contain any aspartyl residues. It is more than possible, however, as mentioned below, that residues of the latter type might have been produced during the manufacture or esterification of the sample of insulin concerned.

The balance (Table 2) between the amide N of the ester and the products representing asparagine and glutamine residues is reasonable, especially if allowance be made as suggested above for the possibility that one of the glutamyl residues is esterified with less readiness than the others. If allowance is made for known losses of amide N, the data given in Table 3 correspond to those derived in a similar way for the other proteins and are in agreement with the known structure of insulin.

There remains the problem of deciding the origin of the lost amide N. Insulin, when reduced and hydrolysed, gave no aspartidiol, but *O*-acetylinsulin methyl ester, prepared with diazomethane without loss of amide N, gave 0·05 group/mole, and this must have come from a *C*-terminal aspartic acid residue which, in its turn, must represent a *C*-terminal asparagine residue which had lost its amide N. By difference therefore the remaining loss of 0·13 group of amide N/mole of insulin (Table 4) must have come, in large part, from asparaginyl residues in the peptide chains. On esterification with methanolic HCl under the usual

Table 4. *Loss of amide nitrogen during the preparation of insulin from ox pancreas and on subsequent esterification with methanolic hydrochloric acid*

Results are expressed as groups/mole (5732 g.).

	Loss of amide-N		
	1	2	3
	Total	*C*-terminal	In peptide chains (1 – 2)
Insulin (see text)	0·18	0·05	0·13
Insulin ester (see text)	0·55	0·10	0·45
Loss on esterification with methanolic 0·1 N-HCl	0·37	0·05	0·32

conditions there was a further loss of amide N and this was derived, as before, from both types of asparagine residues. These deductions suggest strongly that the specially prepared sample of insulin used in our research had lost 0·18 group of amide N/mole through hydrolysis during its preparation from the frozen pancreas, which involves, at various stages, an acid medium. It has already been shown that the hexa-amide (*A* peak) insulin of Harfenist & Craig (1952) contains the theoretical amount of amide N. From the discussions given above it would seem clear that their penta-amide (*B* peak) material is not a chemical entity, but an artifact of hydrolysis during preparative treatment in which all of the asparagine residues in the molecule have become modified to some extent.

SUMMARY

1. A method for estimating the total number of asparagine and of glutamine residues in proteins has been explored.

2. The method is based on modifications which aspartyl, glutamyl, *C*-terminal asparaginyl and *C*-terminal glutaminyl residues undergo when esterified and then reduced with lithium borohydride.

3. After hydrolysis the two latter residues are represented by γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid respectively.

4. Asparaginyl and glutaminyl residues in the peptide chains do not become modified and after hydrolysis can be estimated as aspartic and glutamic acids respectively.

5. Interference due to reductive cleavage of peptide bonds occurs to a very small extent and can be allowed for.

6. Data are presented for the asparaginyl, glutaminyl, aspartyl and glutamyl residues in ox insulin, β- and β_1-lactoglobulin and lysozyme.

7. δ-Hydroxy-γ-aminovaleric acid and γ-hydroxy-β-aminobutyric acid have been synthesized and their properties are described.

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Structure of the Polyglutamic Acid from *Bacillus subtilis*

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The structure of the polyglutamic acid produced by certain strains of the genus *Bacillus* has been studied by a number of methods (literature reviews: Bricas & Fromageot, 1953; Waley, 1955). Bruckner & Ivanovics (1935) isolated and characterized the capsular substance of *B. anthracis* and showed that it consisted largely of units of D-glutamic acid in peptide linkage. More recent work on the soluble peptide from *B. subtilis* by Bovarnick (1942), the *B. anthracis* capsule by Hanby & Rydon (1946) and the soluble peptide from *B. mesentericus* by Pongor (1950) has shown that, to the extent of at least 99%, glutamic acid is the only nitrogen-containing substance in the peptide hydrolysates. The optical configuration of the glutamic acid appears to vary from one preparation to another although it is largely of the D form.

The molecular weight of the peptide depends on the method of preparation and values ranging from 5000 to 70 000 have been reported. The workers concerned, nevertheless, have not found the determination of molecular weight to be very straightforward. Physical measurements such as sedimentation and diffusion require much care as the results are markedly dependent on pH and salt concentration, owing to the poly-electrolyte nature of the material (Bovarnick, Eisenberg, O'Connell, Victor & Owades, 1954). Amino end-group analysis by the Sanger technique (Waley, 1955) is not very precise, for it is known that the reaction between a terminal glutamic acid residue and dinitrofluorobenzene can be taken to completion only with difficulty. Amino nitrogen determination with nitrous acid (Hanby & Rydon, 1946) is subject to the uncertainty of the behaviour of γ-peptide linkages with the reagent (Chibnall & Westall, 1932; Sachs & Brand, 1954).

The nature of the peptide linkage between the glutamic acid residues calls for more extended comment. Bovarnick (1942) concluded that the peptide from *B. subtilis* must be largely γ-linked on the basis of a negative biuret test and the absence of racemization on standing in alkaline solution. From titration data Hanby & Rydon (1946) concluded that both α- and γ-linkages were present in the capsular substances from *B. anthracis*. Kovacs & Bruckner (1952) applied the Curtius degradation and Bruckner, Kovacs & Nagy (1953) the Hofmann degradation to the peptide from *B. subtilis*. In both cases the presence of γ-linkages was confirmed by the isolation of crystalline derivatives of β-formylpropionic acid. No evidence for $\alpha\gamma$-diaminobutyric acid, indicating an α-linkage, was obtained, but the complete absence of this substance was not demonstrated even though it could be easily prepared by both degradation techniques from samples of synthetic poly-α-glutamic acid (Kovacs, Bruckner & Kovacs, 1953; Bruckner, Kovacs & Kovacs, 1953). Waley (1955) compared the natural polyglutamic acid with synthetic α-linked and γ-linked glutamyl peptides in respect to solubility in water, reaction with ninhydrin, titration constants, rate of acid hydrolysis and infrared spectra, and has concluded that the natural material most closely resembles the synthetic γ-linked material. The evidence thus suggests that the polypeptide is largely, if not wholly, γ-linked; nevertheless, the presence of a small percentage of α-linkages has not been completely ruled out and the possibility that branch-peptide chains might occur has not been the subject of any specific inquiry.

The work reported in the present paper has been undertaken in the hope that the procedure of reduction of the ester with lithium borohydride would provide a final answer to these queries. The problem is really no more than a special, if somewhat simplified, example of the more general one of amide distribution dealt with successfully in the preceding paper (Chibnall, Haselbach, Mangan & Rees, 1958*a*). On the assumption that the polypeptide molecule consists of one peptide chain built up of glutamic acid residues, the ester should give δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV) if these are γ-linked, α-hydroxy-γ-aminovaleric acid (α-γ-HAV) if they are α-linked, and in both glutamidiol from the *C*-terminal residue. If, on the contrary, a reductive fission (Crawhall & Elliott, 1955) of the polypeptide itself should occur then any hydroxy-amino acid produced would have the reverse significance; δ-γ-HAV would denote an α-linkage

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and δ-α-HAV a γ-linkage. In the ester, both types of linkage would lead to an increase in amount of glutamidiol. The results show that the glutamic acid residues are exclusively γ-linked. The presence or absence of branched chains and rings has been investigated by titration. If the product is a simple peptide chain of glutamic acid residues, one of them being *C*-terminal, the total number of titratable groups will be equal to the number of residues plus one. If branched chains should be present the number of *C*-terminal residues/mole would be correspondingly increased, but the total number of titratable groups/mole would remain unchanged. On the other hand, intramolecular folding to form closed rings, of the type that Ivanovics & Horvath (1953) suggest may be present in the parent capsular material of *B. anthracis*, would lead to a corresponding reduction in the total titratable groups/mole. The results suggest that branched chains and rings are not present.

EXPERIMENTAL AND RESULTS

Esterification

Esterification. The methods of esterification and of estimation of methoxyl are those given by Chibnall, Mangan & Rees (1958*c*).

Polypeptide from B. subtilis. Sample *A* (reputed mol.wt. 13 000) and sample *B* (reputed mol.wt. 39 000) were kindly provided by Dr M. Bovernick.

Synthetic poly-α-glutamic acid. This was kindly supplied by Dr J. Watson (Hanby, Waley & Watson, 1950), who informed us that the sample had been prepared from the ester by saponification with ethanolic KOH.

Estimation of δ-hydroxy-γ-aminovaleric acid. As the hydrolysate of the reduced bacterial peptide ester contained only glutamic acid and products derived from it, we assumed in the early stages of this work that a direct determination of the formaldehyde given on periodate oxidation (Rees, 1958) would be a valid measure of the total amount of δ-γ-HAV and glutamidiol present. Misleading deductions were indeed drawn as to the extent of reduction achieved before it was realized that the colour intensity on which the estimation was based could be lowered by the presence in the hydrolysate of boric acid derived from the reducing agent. The magnitude of the effect is illustrated by the following experiment. A suitable hydrolysate, prepared from a sample of peptide ester that had been reduced with a tenfold molar excess of $LiBH_4$, was taken and the formaldehyde formed on treatment with periodate was estimated on a sample. δ-γ-HAV equivalent to the formaldehyde found was then added to another sample and formaldehyde was again determined. The recovery of the hydroxyamino acid was only 83·3%. In another experiment, with an ester reduced with a 15-fold molar excess of $LiBH_4$, the recovery was 87%. To overcome such irregular interference an attempt was made to remove the boric acid as volatile methyl borate. In a trial experiment three 1 ml. samples of a solution of serine (13·7 mg. in 50 ml.) were treated in the following way. The first was evaporated to dryness *in vacuo* at room temperature. To the second was added 6·8 mg. of $LiBH_4$ in tetrahydrofuran (1 ml.). Excess of HCl was then added and the mixture evaporated to dryness *in vacuo* at room temperature. A similar amount of $LiBH_4$ in tetrahydrofuran was added to the third. The mixture was then heated with excess of methanolic HCl on the water bath for 3–4 min. and then evaporated to dryness *in vacuo*. The treatment with methanolic HCl was thrice repeated. Periodate–formaldehyde estimations on the three samples gave recoveries of 100, 44 and 99·8% serine respectively, showing that the estimation of serine, like that of δ-γ-HAV, is unreliable in the presence of boric acid. The latter can, however, be effectively removed as volatile methyl borate. Unfortunately, the modified procedure cannot be applied to the estimation of δ-γ-HAV as ring formation, to give pyrrolidonyl alcohol occurs to a small extent during the treatment with methanolic HCl. In the present work δ-γ-HAV has been estimated by column analysis.

Column analysis of glutamic acid, δ-hydroxy-γ-aminovaleric acid, δ-hydroxy-α-aminovaleric acid and glutamidiol. The method of Chibnall *et al.* (1958*a*) was used. In the absence of aspartic acid and its derivatives no difficulty was experienced in obtaining a good separation between the two hydroxyamino acids. As glutamidiol was the only amino alcohol concerned it could be determined by prolonging the elution until the fourth day.

Stability of δ-hydroxy-γ-aminovaleric acid and δ-hydroxy-α-aminovaleric acid on acid hydrolysis. As unexpectedly large amounts of dissolved NH_3 were found in the hydrolysates of the reduced bacterial polypeptide it was necessary to investigate the stability of the two hydroxyamino acids under similar conditions of hydrolysis. The recovery of δ-γ-HAV, ascertained by estimation of periodate–formaldehyde, was 85% before column analysis (Table 1) and 72·5% after it (Chibnall *et al.* 1958*a*). Likewise the recoveries of δ-hydroxy-α-aminovaleric acid (δ-α-HAV),

Table 1. *Stability of δ-hydroxy-γ-aminovaleric acid and δ-hydroxy-α-aminovaleric acid on acid hydrolysis*

Acid used: 10N-HCl–formic acid (1:1, v/v).

	δ-γ-HAV		δ-α-HAV		
Period of hydrolysis (hr.)	Equiv. periodate–formaldehyde (as % equiv. total N)	Ammonia N (as % of total N)	δ-α-HAV N (as % of total N)	Proline N (as % of total N)	Ammonia N (as % of total N)
3	91·3	1·78	—	—	—
6	87·0	2·32	—	—	—
24	85·0	3·33	65·7	2·30	2·21

estimated with ninhydrin, were 65·7% before column analysis and 60% after it. Proline, which might be formed from δ-α-HAV by dehydration (Plieninger, 1950) was estimated by the column procedure of Moore & Stein (1954). Ammonia was estimated with ninhydrin by the Conway technique (Chibnall, Mangan & Rees, 1958*b*). As only limited supplies of the two synthetic hydroxyamino acids were available and the amount of NH_3 likely to be produced was small, particular attention was given to the purity of the reagents used. Redistilled constant-boiling HCl was brought to 10N with gaseous HCl; formic acid was redistilled. The saturated K_2CO_3 solution was aerated for some time to remove NH_3. The hydrolysis of the samples was carried out in a flask fitted with a long narrow air condenser to minimize adsorption of atmospheric NH_3. In the Conway estimation the hydrolysis blank was 0·15 μg., the apparatus blank was 0·46 μg. and the NH_3 measured was not less than 3·4 μg.

Comment. The data given in Table 1 show that both hydroxyamino acids undergo a slow change on hydrolysis. In each case ammonia is produced, but in amount much less than the equivalent of parent acid lost, or than might have been expected from the analysis of a reduced bacterial polypeptide hydrolysate. δ-γ-HAV must undergo ring formation to give pyrrolidonyl alcohol, whereas δ-α-HAV, which gives but very little proline, must go over to the δ-lactone. Under the conditions of hydrolysis and column analysis employed in the present work these changes, though of relatively large magnitude, were readily reproducible, and we have confidence in the correction factors used in the present as well as in previous papers (Chibnall & Rees, 1958; Chibnall *et al.* 1958*a*).

Titratable carboxyl groups of the bacterial peptide

On the assumption that sample *A*, of reputed mol.wt. 13 000, is a simple peptide chain bearing a *C*-terminal glutamic acid residue, it will contain 99 residues (or N atoms) and 100 titratable groups/mole or, expressed in another way, the titration value will be equivalent to 101% of total N. Likewise, sample *B* of reputed mol.wt. 39 000 would have a titration value equivalent to 100·3% of total N.

In a typical experiment sample *A* (35 mg.) was suspended in water (2 ml.) and brought to pH 7 (bromothymol blue) with 0·0143N-NaOH. The material dissolved during the operation and the end point was judged by colour comparison with a similar volume of buffer solution, pH 7, contained in a flask of like dimensions. The solution was then made to standard volume and total N determined. The titration value was equivalent to 94·5% total N and was easily reproducible to within 0·5%. Sample *B* gave a titration equivalent to 97·0% of total N. Under comparable conditions glutamic acid was fully titrated.

The values given by both samples of polypeptide were less than the theoretical minimum for a single or branched-chain molecule, and for reasons discussed below it was considered that this might be due to slight esterification brought about by the use of alcohol during the preparation of the samples from the bacterial capsule. Methoxyl determinations were accordingly made and these were found to be equivalent to 5·0 and 2·95% of total N for samples *A* and *B* respectively; each value remained unchanged if the material was twice dissolved in water and dried from the frozen state to remove any excess of methanol. Adding these values to the titration values, both products show a close agreement between the number of carboxyl groups and the number of residues/mole, indicating the probable absence of ring structures.

Esterification of the bacterial polypeptide

According to our experience, proteins, when suspended in methanol and treated with methanolic HCl of the requisite strength, will dissolve readily as the hydrochloride and full esterification can be achieved under suitable conditions (Chibnall *et al.* 1958*a*). The bacterial polypeptide and also synthetic poly-α-glutamic acid, however, will not disperse when treated in this way, presumably owing to the large excess of carboxyl groups (cf. Hanby *et al.* 1950). The following procedure was found to be effective for the purpose.

The bacterial polypeptide or the poly-α-glutamic acid (125 mg.) was suspended in water (1 ml.) and 0·96N-LiOH added with stirring until the product had dissolved (0·825 ml.). The clear solution was then freeze-dried. The residue, a hard mass, was ground up with methanolic HCl equivalent to the LiOH added, when solution was effected in about 3 min. By the appropriate addition of methanolic 0·94N-HCl and methanol the volume was then brought to 25 ml. and the HCl to 0·1N or 0·05N as the case may be. After the solution was brought to 25° a sample (2 ml.) was withdrawn and run into acetone–ether (1:1; 12 vols.). The precipitated material was centrifuged off, washed once with the same volume of acetone–ether and then dissolved in water (8 ml.). The extent of esterification was ascertained by titration, as described in the preceding section, or by Zeisel analysis. The main solution was kept at 25°, and further samples were withdrawn at intervals for treatment in the same way.

Comment. The data given in Table 2 show that the γ-carboxyl groups of the poly-α-glutamic acid are more reactive than the carboxyl groups of the bacterial polypeptide; indeed, the former product is more than 90% esterified in only 10 min. That γ-glutamyl groups should esterify with such great rapidity was unexpected, because in a more comprehensive investigation of β-lactoglobulin, with a similar esterifying medium, only 69·1% of such residues were esterified in 30 min. and 86·6% in 1 hr. (Chibnall *et al.* 1958*c*), a reaction rate which is impressive and greater than that for the bacterial polypeptide, but much lower than that for the poly-α-glutamic acid.

One other observation needs mention. About 93–94 % esterification of the bacterial polypeptide occurs in about 3 hr. with methanolic 0·1 N-HCl and the product remains soluble in the medium. When precipitated by acetone–ether, as described above, the centrifuged and washed material dissolved readily in water. If, on the contrary, the period of esterification was extended beyond about 3 hr. there was a slow separation of the fully esterified material, and this, when dried as before, was no longer soluble in water. The significance of the difference in behaviour will appear later.

Reduction of the bacterial polypeptide and its methyl ester

The results are given in Table 3. Experience has shown that a tenfold molar excess of $LiBH_4$ was the most suitable for present purposes, but under certain conditions discussed below it was found difficult to effect complete reduction of the peptide ester, even with a much greater excess of reductant than this. The degree of dispersion of the polypeptide and ester was one of the factors concerned, and this was controlled in the following way.

The polypeptide (25 mg.) suspended in water (0·5 ml.) was treated with sufficient aq. LiOH soln. to effect solution. It was then precipitated by adding methanol (20 ml.) containing 10 % (w/v) of LiCl. The product was washed with acetone (20 ml.), then three times with dry ether (20 ml.) and finally three times with tetrahydrofuran (12 ml.). During treatment the polypeptide was in contact with methanol for 3–5 min. The ester hydrochloride (0·1265 g.), prepared as described earlier, was washed first with ether and then three times successively with tetrahydrofuran (7 ml.) and finally brought to a fine suspension in that solvent. The polypeptide, as a fine suspension in

Table 2. *Esterification of polyglutamic acids in methanolic hydrochloric acid at 25°*

The bacterial polypeptide was sample *A*, of reputed mol.wt. 13 000 (see text). Progress of esterification was measured by titration or, where marked *, by Zeisel determination of methoxyl. Results are expressed as percentage total esterification.

Time after addition of methanolic HCl (min.)	Esterification of bacterial polypeptide in: Methanolic 0·1 N-HCl	Methanolic 0·05 N-HCl	Esterification of synthetic poly-α-glutamic acid in methanolic 0·1 N-HCl
0	10·2	10·2	27·0
10	—	—	91·6
20	—	—	96·7
30	49·4	44·5	98·0*
60	69·4	65·1	—
90	78·9	—	—
120	86·6	80·0	—
165	92·5	—	—
240	—	92·0	—
1080	98·0, 97·0*	—	—

Table 3. *Analytical data for polyglutamic acids and their methyl esters, with details of reduction with lithium borohydride*

The reputed molecular weight of sample *A* of the bacterial peptide was 13 000, and that of sample *B* 39 000 (see text).

Expt. no.	Bacterial peptide (sample *B*) 1	2	3	Bacterial peptide methyl ester (sample *B*) 4	5	6	7	8	Bacterial peptide methy ester (sample *A*) 9	10	Synthetic poly-α-glutamic acid methyl ester 11
Methoxyl (% by wt.)	2·95	2·95	2·95	93·8	98·0	—	—	—	—	97·0	—
Titratable carboxyls (% total carboxyl groups)	97·0	97·0	97·0	—	—	—	—	—	7·3	—	—
$LiBH_4$ (mol./mol. of peptide N)	—	—	10	15	10	8	9	19	10	10	8
Molarity of $LiBH_4$	—	—	0·33	0·5	0·33	0·5	0·6	1·2	0·33	0·33	0·5
Reduction time (hr.)	—	—	6	6	6	5	18	94	6	6	18
Hydrolysis time (hr.)	2	24	3	16	6	2	2	2	7	6	6

tetrahydrofuran (1 ml.), or a sample of the ester suspension (1 ml. = 25·3 mg.), was then reduced under reflux for 6 hr. with $LiBH_4$ (33 mg.) in a total volume of 3 ml. of tetrahydrofuran. At the end of the reaction the cooled mixture was treated with excess of methanolic HCl and allowed to stand overnight. The time interval is advantageous in that borate complexes are thereby decomposed and most of the borate passes into solution. The reduced product was then centrifuged off, washed with ether and dried *in vacuo*. It was hydrolysed with 10N-HCl–formic acid (1:1, v/v; 5 ml.) for the required time.

DISCUSSION

The bacterial polypeptide itself (Table 4, no. 3) exhibited but little change on reduction. The data given in Table 4 show that in all the experiments the amount of glutamidiol given by the polypeptide ester is in excess of that which could arise from the presence of 1 *C*-terminal residue/mole (0·33% for sample *A*, 1% for sample *B*). As was mentioned earlier, the finding could be interpreted as evidence for branch-peptide chains, each of which would carry a *C*-terminal residue. Under such conditions one would expect to find that the amount of glutamidiol produced was more or less independent of the reductive conditions employed and this is definitely not the case. The diol production is clearly a function of both reductant concentration and reaction time, and was due, almost certainly, to reductive cleavage.

In none of the experiments with bacterial material quoted in Tables 3 and 4 was δ-α-HAV encountered, and an amount representing less than 0·05% could have been readily detected. As this particular hydroxyamino acid can undergo dehydration on acid hydrolysis to give proline a sample of a reduced ester from sample *B* was analysed for this amino acid on a standard ion-exchange column (Moore & Stein, 1954); none was found. It may be concluded therefore that α-glutamyl peptide linkages do not occur in either sample of bacterial polypeptide, and the possible small excess of δ-γ-HAV produced on reduction of the polypeptide itself must be due to slight esterification during the initial treatment to disperse the product or, less likely, to a direct reduction of the α-carboxyl group.

The evidence for the presence of γ-glutamyl peptide linkages on the other hand is unequivocal, as shown by the extensive production of δ-γ-HAV. Nevertheless, in the early stages of the work certain of the reduced ester hydrolysates gave results which were not readily interpretable in terms of a single-chain poly-γ-glutamyl molecule. Two unusual features of sporadic and irregular occurrence puzzled us, and until these had been clearly defined and their full import realized, much effort was wasted in trying to obtain concordant results with

Table 4. *Amino acid analysis of polyglutamic acids and their methyl esters after reduction with lithium borohydride*

	N (% of total N)										
Expt. no.	1	2	3	4	5	6	7	8	9	10	11
Amino N after hydrolysis*	98·0	96·0	96·2	91·3	82·2	—	—	—	—	—	90·3
Glutamic acid	—	—	92·5	8·0	20·0	22·4	6·6	2·9	5·5	22·0	5·7
δ-γ-HAV	—	—	3·6	82·2	60·5	57·8	72·5	64·3	83·7	63·1 (0·85)†	4·3
δ-α-HAV	—	—	0	0	0	0	0	0	0	0	77·8
Glutamidiol	—	—	0	2·0	1·2	—	1·8	3·9	2·7	1·4	3·0
NH_3	0	0·4	1·3	6·0	14·1	—	—	—	7·0	9·4	1·3
Total accounted for	—	—	97·4	98·2	95·8	—	—	—	98·9	95·9	92·1

* Corrected for NH_3 present.

† Representing *N*-terminal δ-γ-HAV in peptides, see text.

Hydrolysis time (hr.) 2 24 3 16 6 2 2 2 2 7 6 6

reduced products prepared under what were thought at the time to be comparable conditions. First, the extent of reduction appeared to be unpredictable and was not brought under control until its dependence on the degree of esterification had been elucidated. Paradoxically, both sample *A* and sample *B* under comparable conditions underwent less reduction when fully esterified than when esterified to only about 93% (Table 4, nos. 5 and 10, contrasted with nos. 4 and 9). Secondly, the ammonia produced on hydrolysis of the reduced esters varied in the same irregular way and was also shown to be subject to the degree of esterification. In this case, however, the fully esterified product gave more than that esterified by only 93%.

We believe that these anomalies are a reflexion of the state of aggregation of the products concerned. Where less than full esterification has been achieved ionizable polar groups are still present. Presumably the individual peptide chains retain their intrinsic property of free movement and in an aqueous acid medium H^+ ions will have ready access to the peptide bonds. It is thus not surprising that such a product should be soluble in water and readily hydrolysed by aqueous acid. Under the usual conditions the polypeptide itself is fully hydrolysed in 2 hr., whereas 3 hr. was sufficient if it had been esterified to 93% before reduction. In the fully esterified product, on the contrary, the *N*-terminal residue carries the only polar group. It is to be expected therefore that the long peptide chains will tend to aggregate under the influence of van der Waals forces and will be insoluble in water. The approach of H^+ ions to the peptide bonds may thus be hindered and hydrolysis in aqueous acid slowed. It has, indeed, been observed that under the usual conditions of hydrolysis peptides containing *N*-terminal δ-γ-HAV residues which will react with periodate are still present in small amounts after 6 hr. On column analysis they emerge immediately behind δ-γ-HAV itself (Table 4, no. 10). It is more difficult to argue about susceptibility to reduction in a non-aqueous medium, but the results given in Table 4 are consistent with the view that the approach of the reducing agent is the more difficult when the product is fully esterified.

All of these observations are in keeping with the suggestion that the fully esterified product, both before and after reduction, has a more closely knit configuration than the one that is esterified to a lesser degree, and accordingly may require more drastic treatment than usual with strong acid to fragment it. It is to be noted that during the hydrolysis of the reduced ester the derived δ-γ-HAV has undergone more extensive decomposition, with production of ammonia, than when present as the free hydroxyamino acid in the hydrolysis medium (Table 1), and this is especially marked in the fully esterified products. The evidence thus seems fairly conclusive that the samples of bacterial polypeptide, of high as well as of low molecular weight, are single-chain poly-γ-glutamyl peptides.

The reduction of methyl poly-α-glutamate (Tables 3 and 4, no. 11) was allowed to proceed for 18 hr. to emphasize any reductive cleavage and the presence of glutamidiol in excess of that derived from one *C*-terminal residue shows that this has indeed occurred to a small extent. The production of 4·3% of δ-γ-HAV suggests that the sample of γ-ethyl glutamate used in the synthesis of the poly-α-glutamic acid contained about 5% of the α-homologue. In this experiment proline was not determined, and the omission is in part responsible for the rather low recovery of nitrogen accounted for.

Finally, there are two observations arising out of the present study which need particular emphasis, for they undoubtedly have implications in general protein chemistry. The first is the ease with which γ-carboxyl groups in a long peptide chain will esterify. The second is the clear demonstration that the destruction of residues in the early stages of hydrolysis, when they are still in peptide linkage, may greatly exceed that of the free amino acid under the conditions used for hydrolysis (cf. Martin & Synge, 1945).

SUMMARY

1. Titration of two different samples of polyglutamic acid from *Bacillus subtilis* suggests that they are built up exclusively of open peptide chains, and that closed ring structures are not present.

2. When esterified and reduced with lithium borohydride, the products given on subsequent acid hydrolysis show that the glutamic acid residues in the polypeptide are exclusively γ-linked and that the presence of branch chains is unlikely.

3. The properties of the ester, the extent of reduction achieved and the composition of the hydrolysate of the reduced ester are dependent in an unusual way on whether the degree of esterification is less than 93% or exceeds this value.

4. It is suggested that this difference in ester behaviour can be correlated with the number of residual carboxyl groups present.

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Determination of the *N*-Terminal Residues in Proteins with Methoxycarbonyl Chloride

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The well-known procedure of Sanger (1945), which employs 1-fluoro-2:4-dinitrobenzene (FDNB) for the determination of *N*-terminal residues in proteins, has been widely used, but in certain cases, e.g. tropomyosin, where the presence of a *C*-terminal residue can be demonstrated by means of carboxypeptidase, it has so far failed to reveal the presence of any *N*-terminal residue at all. This apparent conflict of evidence led Bailey (1953) and Desnuelle (1953) to suggest tentatively that the *N*-terminal amino group might be linked with an ω-carboxyl group in the peptide chain to give a structure which can be schematically described as a 'figure 6'. Bailey recognized, however, that the failure to detect *N*-terminal residues with FDNB might be due either to steric factors or to the instability of the derived dinitrophenyl-(DNP)-amino acid given on subsequent acid hydrolysis. There is one other possibility. When a protein is treated with FDNB under the usual conditions the DNP-protein rapidly comes out of solution. Tropomyosin is rich in lysine residues, and it might well be that these react to give an insoluble product before the *N*-terminal residues have become fully substituted.

To probe these possible limitations to the use of FDNB an alternative reagent has been sought which would fulfil the following three conditions. First, it should be of low molecular weight so as to reduce the possibility of steric hindrance. Secondly, it should condense with proteins to give products which remain soluble in the reaction mixture, thereby favouring the attainment of full substitution. Thirdly, the substituted protein should be readily soluble in concentrated hydrochloric acid at a temperature around 37°. To effect solution of DNP proteins it is generally necessary to treat with boiling 6N-hydrochloric acid for some time. Such drastic conditions of hydrolysis, however, might not be necessary to split off the *N*-terminal residue, for the stability of the peptide bond concerned would be lowered by the elimination of the strongly charged terminal amino group. It was hoped that hydrolysis with concentrated hydrochloric acid at 37° over a period of several days (Gordon, Martin & Synge, 1941) would be suitable for the purpose, and thus permit the employment of a reagent giving an *N*-substituted amino acid which might not survive prolonged boiling in 6N-hydrochloric acid.

Methoxycarbonyl (MC) chloride (I), which readily condenses with an amino acid (II) to give the methoxycarbonylamino acid (III), was found to satisfy these conditions.

$$\underset{\text{(II)}}{HO_2C\cdot CH(R)\cdot NH_2} + \underset{\text{(I)}}{Cl\cdot CO\cdot OCH_3} \rightarrow \underset{\text{(III)}}{HO_2C\cdot CH(R)\cdot NH\cdot CO\cdot OCH_3} + HCl$$

Methoxycarbonyl chloride (MC-Cl) was first used to prepare MC-glycine by Leuchs (1906) and the homologous reagent ethoxycarbonyl chloride was used extensively by Fischer (1906) in his early synthetic work on peptides. All the MC-amino acids investigated in the present research were readily decomposed in dilute boiling hydrochloric acid, but they were relatively stable in 8·6N-hydrochloric acid at 37°. Also, as was expected, the peptide bond in MC-leucylglycine was much less stable than in the dipeptide itself and was readily cloven by 8·6N-hydrochloric acid at 37° (Table 1). These observations form the basis of a procedure for the determination of the *N*-terminal residues in proteins which is explored in the present paper.

For explanation of the abbreviations used for amino acids in this paper see *Biochem. J.* 1952, **52**, 1.

EXPERIMENTAL AND RESULTS

Materials and methods. The samples of ox insulin (Boots Pure Drug Co., Ltd., Nottingham, batch no. 9011G) and β-lactoglobulin (sample A) have already been described (Chibnall, Mangan & Rees, 1958). Samples of ovalbumin and rabbit tropomyosin were prepared by Dr Kenneth Bailey.

Methoxycarbonyl chloride was obtained from L. Light and Co., Wraysbury.

Except when otherwise stated, the ninhydrin method of Yemm & Cocking (1955) was used to estimate α-amino N. When the Van Slyke procedure for amino N was employed the reaction time was 5 min. with amino acids and 15 min. with proteins.

Synthesis of methoxycarbonylamino acids

Methoxycarbonylglycine. This was prepared by the method of Meyer & Go (1934).

Methoxycarbonyl-DL-alanine. DL-Alanine (1·8 g.) and Na_2CO_3 (1·6 g.) were dissolved in N-NaOH (20 ml.) and cooled to 0°. MC-Cl (2·3 ml.) in portions of 0·25 ml. was then added during the course of 2 hr. with stirring, the alkalinity being maintained at pH 9 with N-NaOH. At the end of the reaction the mixture was acidified with HCl, evaporated to dryness *in vacuo* and the residue extracted with ether under reflux. The ethereal solution was reduced in volume and excess of light petroleum added. MC-DL-alanine crystallized on standing and was recrystallized from ether–light petroleum. Yield 70% (Found: N, 9·54. $C_5H_9O_4N$ requires N, 9·52%).

Methoxycarbonyl-L-valine, -L-leucine and -DL-leucine. These were prepared by the method of Petri & Staverman (1952) and recrystallized from ether–light petroleum. Yields 78, 80 and 70% respectively. The last-named stood for several weeks at 0° before it crystallized.

Methoxycarbonyl-L-isoleucine. This was prepared by the method used for the DL-product by Hanby, Waley & Watson (1950), who obtained an oil. The L-isomer crystallized readily from ether–light petroleum. Yield 82% (Found: N, 7·41. $C_8H_{15}O_4N$ requires N, 7·41%).

Methoxycarbonyl-DL-phenylalanine. This was prepared as an oil by the method of Petri & Staverman (1952). In the present case the oil solidified to a white crystalline mass on standing at room temperature.

ε-Methoxycarbonyl-L-lysine. This was prepared in a similar way to the DNP compound (Porter & Sanger, 1948). L-Lysine dihydrochloride (2·2 g.) in water (10 ml.) was brought to pH 10 with N-NaOH and treated at the boil with excess of $CuCO_3$. The mixture was filtered and the residue washed with water. To the dark-blue filtrate was added Na_2CO_3 (0·8 g.) in a little water and then MC-Cl (1·2 ml.) in portions of 0·2 ml. at intervals during the course of 2 hr. with stirring. The precipitate was filtered off, washed successively with water, ethanol and ether and dried. It was then dissolved in water containing just sufficient N-HCl to give a clear solution, and copper was removed with H_2S. A trace of charcoal was then added and the mixture filtered. The clear filtrate was taken to dryness *in vacuo*, dissolved in ethanol and light petroleum added in excess. On standing overnight ε-MC-L-lysine hydrochloride crystallized out and was recrystallized from ethanol–light petroleum. Yield 70% (Found: N, 11·66. $C_8H_{16}O_4N_2,HCl$ requires N, 11·64%). Amino N (Van Slyke) 50·1% of total N.

Methoxycarbonyl-DL-aspartic acid, methoxycarbonyl-DL-glutamic acid and α-ε-bis-methoxycarbonyl-L-lysine. These were prepared by the same procedure as MC-DL-alanine. The first crystallized readily from acetone–light petroleum. Yield 65% (Found: N, 7·30. $C_6H_9O_6N$ requires N, 7·33%). The last two were oils which could not be characterized.

Methoxycarbonyl-L-leucylglycine. This was prepared by the procedure of Fischer (1905) for the corresponding ethoxycarbonyl compound. Yield 60% (Found: N, 11·28. $C_{10}H_{18}O_5N_2$ requires N, 11·38%).

Stability of methoxycarbonylamino acids to acid hydrolysis

All the above-mentioned MC-compounds when treated with ninhydrin gave less than 1% of the colour for the amino acid itself except the monolysine derivative, which gave 49·5%.

Standard solutions of the MC-amino acids were prepared and fractions treated with boiling acid under reflux or in a sealed tube at 100°, as noted in Tables 1 and 2. After removal of the excess of mineral acid *in vacuo* at room temperature the amount of decomposition of the MC-amino acid to give the parent amino acid was estimated with ninhydrin. With MC-leucylglycine, where cleavage of two bonds to give α-amino N is possible, it was convenient to represent the maximum fission as 200%.

The results given in Table 1 show that the MC-amino acids are fairly readily decomposed by hot strong mineral acid, but that the effect is markedly and progressively reduced as the strength of the mineral acid is decreased. The dipeptide bond in MC-leucylglycine is almost completely cloven in 0·5N-HCl during 18 hr. at 100° with a loss of less than 20% of the resulting MC-leucine. The observation was encouraging, but experience has since

Table 1. *Hydrolysis of methoxycarbonylglycine and methoxycarbonyl-leucylglycine under various conditions*

A, MC-glycine; *B*, MC-leucylglycine. Results are expressed as percentage fission of one methoxycarbonyl (or peptide) bond.

Time of hydrolysis (hr.)	Hydrolysis medium: 10 N-HCl–formic acid (1:1, v/v) at b.p.		2·3 N-HCl at b.p.		1·1 N-HCl at b.p.		0·5 N-HCl at 100°		0·25 N-HCl at 100°	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
0·5	—	—	—	—	4	37	—	—	—	—
1·0	—	—	—	—	6·5	53	—	—	—	—
2·0	—	—	25·5	113	—	—	—	—	—	—
4·0	96	169	—	—	—	—	—	—	—	—
18·0	98	200	—	—	—	—	17	114	6	75

Table 2. *Hydrolysis of various methoxycarbonylamino acids by 8·6 N-hydrochloric acid at 37°*

Results are expressed as percentage fission of one bond.

Time (days)	MC-glycine (%)	MC-leucine (%)	MC-leucyl-glycine (%)	MC-valine (%)	MC-iso-leucine (%)	MC-phenyl-alanine (%)	ε-MC-lysine (%)
10	12·5	14·2	113·0	9·0	8·0	12·5	63*
21	15·6	20·1	—	—	—	18·3	—

* Determined by the method of Van Slyke (see text).

shown that a much lower temperature is preferable even though the time of hydrolysis is thereby increased. The results given in Table 2 show that with the exception of ε-MC-lysine, which cannot arise from an *N*-terminal residue in proteins, destruction of the MC group on hydrolysis with 8·6 N-HCl does not exceed 14% in 10 days and 20% in 21 days at 37°. The cleavage of the dipeptide bond in MC-leucylglycine is complete in 10 days, whereas in the dipeptide itself only 20% occurs in 10 days and 60% in 21 days; both values are based on the ninhydrin estimation arbitrarily referred to glycine. Synge (1945) showed that leucylglycine was more stable than many other dipeptides.

Chromatography of methoxycarbonylamino acids

Attempts to use ion-exchange columns for the separation of MC-amino acids were not successful. On elution with various strengths of HCl the MC-amino acids emerged well ahead of aspartic acid, but when the procedure was applied to the hydrolysis products of MC-proteins the peaks representing the MC-amino acids were contaminated with acidic peptides. An indicator column of silica gel with alphamino red R as indicator (Tristram, 1946) was also investigated, but perhaps because buffering was not possible no effective separation was achieved except into 'fast'- and 'slow'-moving bands. A buffered column of Celite, subjected to gradient elution (cf. Alm, Williams & Tiselius, 1952) with chloroform and a continuously increasing concentration of ethanol, has given more satisfactory results.

All the operations connected with the column chromatography, including the preparation of the solvents, were carried out in a room maintained at 27°.

Celite. A sample of Celite Analytical Filter-Aid L 665 (Johns–Manville Co. Ltd., London, S.W. 1) was washed twice with chloroform–ethanol (60:40), filtered off and dried at 100° overnight.

Stationary phase. NaH_2PO_4 buffer (0·5 M, pH 4·5) was found the most suitable. On elution it gave low titration blanks.

Solvent 1. Chloroform (May and Baker Ltd., laboratory grade; 700 ml.) was shaken vigorously with NaH_2PO_4 buffer (300 ml.) and left to clear for 2 hr. The shaking was twice repeated at intervals of about 2 hr. and after standing overnight the chloroform layer was filtered to remove droplets of the aqueous phase.

Solvent 2. Chloroform (600 ml.) was mixed with ethanol (400 ml.) and shaken with NaH_2PO_4 buffer (400 ml.). The further treatment followed that of solvent 1. Both solvents were freshly prepared for each chromatogram.

Chromatography on Celite. The column was packed into a silicon-treated glass tube, 27 cm. long and 13·5 mm. int. diam., the lower end being supported by a filter-paper disk resting on a perforated silver plate which was held by a constriction in the tube just above the drawn-out tip. Celite (6 g.) was ground with Na_2HPO_4 buffer (3 ml.) added dropwise. A slurry was then made with 50 ml. of solvent 1. This was poured into the glass tube in six successive portions of about 8 ml., each of which was first vigorously stirred with a fine, perforated, stainless-steel disk fitted to a rod to remove air bubbles and then packed down hard with the aid of a thick filter-paper disk. The excess of solvent and paper disk were then removed and the next portion of slurry was treated in a similar way. The prepared column was about 10·5–11·5 cm. long. It was washed with solvent 1 (50 ml.) under pressure from a cylinder of nitrogen.

A mixture of MC-amino acids (1 μmole of phenylalanine, leucine and isoleucine; 3 μmoles of the other amino acids) was dissolved in NaH_2PO_4 buffer (0·2 ml.), Celite (0·4 g.) added and the product thoroughly mixed. The slurry was added to the top of the column, which had above it about 3 ml. of solvent 1. A disk of filter paper was then introduced and the new material packed. The paper disk was not removed. The column tube was then fixed in position over a drop-counting fraction collector and pressure applied from a cylinder of nitrogen. When the 3 ml. of solvent at the top of the column had passed on, the pressure was released and the walls above the column were washed down with three lots (0·5 ml.) of solvent 1. The apparatus for gradient elution was then fitted (Rees, 1958). The lower reservoir (mixing flask) contained solvent 1 (500 ml.) and the upper one (a shaking funnel) solvent 2. Pressure was then applied and adjusted to deliver 1 ml. of effluent every 8 sec.; 3 ml. fractions were collected.

Titration for acidity. Each fraction was titrated in a stream of nitrogen with methanolic 0·005 N-KOH (Agla micrometer syringe, Burroughs Wellcome and Co., London) and bromothymol blue (0·04% in methanol; 2 drops) as indicator. On account of the high proportion of chloroform in the first 25 fractions, it was necessary to add ethanol (5 drops) in order to ensure a sharp change in the colour of the indicator.

α-Amino nitrogen. After titration, the fractions were evaporated to dryness *in vacuo* and then treated with constant-boiling HCl (1 ml.) in a closed tube at 100° for 24 hr. Excess of HCl was removed *in vacuo* and amino N estimated with ninhydrin. Reagent blanks were determined.

Paper chromatography. The amino acids present in the hydrolysed fractions mentioned above were identified by paper chromatography, with phenol–borate buffer (pH 9·2), butanol–acetic acid–water or pyridine–ethyl methyl ketone–water (Wieland & Bauer, 1951).

Comment. The chromatogram given in Fig. 1 shows that a reasonable separation of all the MC-amino acids present in the mixture has been obtained except for those of leucine, isoleucine and phenylalanine. These three products are all very soluble in chloroform, and if it became necessary to

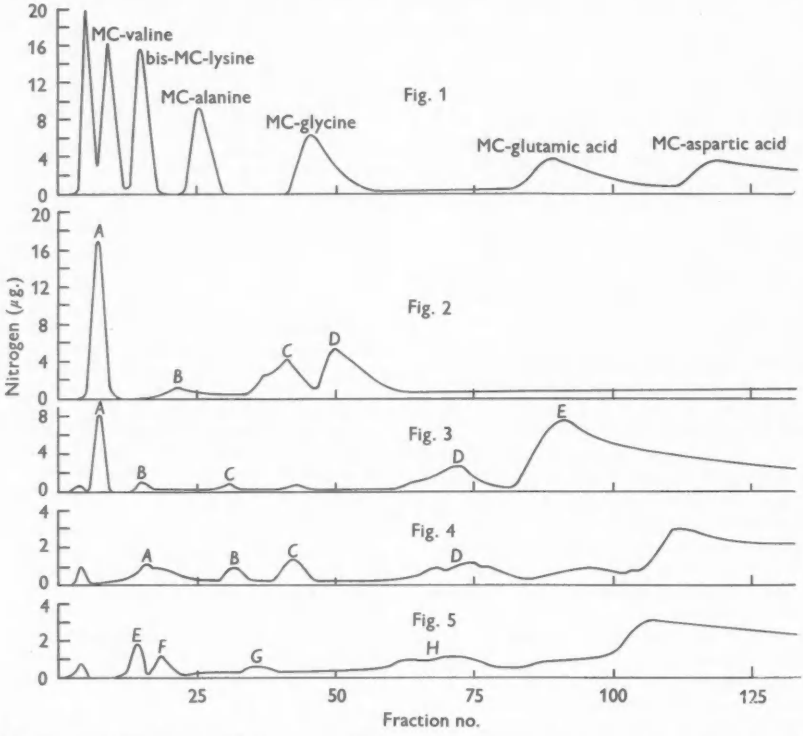

Fig. 1. Chromatographic separation of MC-amino acids on a column of Celite, gradient elution with ethanol–chloroform being used. Fig. 2. Separation of MC-amino acids from a hydrolysate of MC-insulin. Fig. 3. Separation of MC-amino acids from a hydrolysate of MC-β-lactoglobulin. Fig. 4. Separation of MC-amino acids from a hydrolysate of MC-tropomyosin from the rabbit. Fig. 5. Separation of MC-amino acids from a hydrolysate of MC-ovalbumin.

separate them, we believe that this could be achieved either by changing the stationary phase to one more alkaline than pH 7 or, less likely, by using a moving phase in which they are markedly less soluble. In the exploratory application of the procedure to MC-proteins, as described below, both MC-phenylalanine and MC-leucine were encountered. After hydrolysis these were readily characterized as the parent amino acids by paper chromatography. The recovery of MC-amino acids on the Celite column was quantitative.

In any further development of the present procedure two points should be noted. In the first place, pyrrolidonecarboxylic acid moves on the Celite column at the same rate as MC-glutamic acid. It is essential therefore when preparing a hydrolysate of an MC-protein for analysis on the column to prevent pyrrolidone formation, from the (relatively) large amount of glutamic acid present, by adhering to the usual precautions (evaporations at low temperature, etc., cf. Wiltshire, 1953). The point is illustrated in the detailed analysis of MC-lactoglobulin given below. Secondly, ϵ-MC-lysine, which is slow-moving on the Celite column and emerges among the amino acids, undergoes considerable decomposition on treatment with 8·6N-HCl at 37° for 21 days. Bis-MC-lysine moves just behind MC-valine and would be readily detected, but the extent of its decomposition on hydrolysis has not been investigated.

Preparations of methoxycarbonylproteins

Methoxycarbonyl-insulin. Insulin (0·96 g.) was dissolved in a mixture of aq. 60% (v/v) ethanol (60 ml.) and N-NaOH (5·2 ml.). Sodium bicarbonate (0·4 g.) was then added, followed by MC-Cl (0·4 ml.) in 0·05 ml. portions, which were run in slowly with stirring during the course of 3 hr. at room temperature, the alkalinity being maintained at pH 9 with N-NaOH. The reaction mixture was then dialysed, first against distilled water to remove salts, and then against N-HCl, which caused the MC-insulin to precipitate in the dialysis bag. The product was centrifuged, washed with ethanol and ether and dried *in vacuo* over P_2O_5. Yield 96%; determination of free amino N (Van Slyke) showed that 94% of the amino groups had been substituted.

Methoxycarbonyl-β-lactoglobulin. The protein (0·2 g.) was dissolved in 0·25M-$NaHCO_3$ (30 ml.) and treated as above with MC-Cl (0·35 ml.) during the course of 2 hr. After dialysis against distilled water the reaction mixture was evaporated to dryness *in vacuo* in a rotating evaporator below 35°. The product was washed with ethanol and ether and dried *in vacuo* over P_2O_5. Yield 90%.

Methoxycarbonyl-tropomyosin. The protein (0·306 g.) was dissolved in 0·25M-$NaHCO_3$ (30 ml.) and treated as above with MC-Cl (0·6 ml.) during the course of 3 hr.; yield 87%.

Methoxycarbonyl-ovalbumin. The protein (0·225 g.) was dissolved in 0·25M-$NaHCO_3$ (25 ml.) and treated as above with MC-Cl (0·2 ml.) during the course of 2 hr. It was necessary to add 3 drops of capryl alcohol to prevent frothing. Yield 85%; estimation of free amino N (Van Slyke) showed that 90% of the amino groups had been substituted.

Analysis of methoxycarbonylproteins

Hydrolysis. The MC-protein (150 mg.) was treated with 8·6N-HCl (15 ml.) for 21 days at 37°. The insulin and tropomyosin derivatives were spontaneously soluble whereas those of β-lactoglobulin and ovalbumin had dissolved within a few hours. At the end of the period the excess of HCl was removed by repeated evaporation *in vacuo* below 35° and the hydrolysate made up to 5 ml.

Insulin. A portion (1 ml.) of the hydrolysate was evaporated to dryness in a vacuum desiccator. The residue was dissolved in 0·5M-NaH_2PO_4 (0·2 ml.). Celite (0·4 g.) was added and the product well mixed. It was then applied to the Celite column as described previously. Titration of the effluent fractions gave a chromatogram with four peaks (Fig. 2*A–D*). The titrated fractions representing these individual peaks, and also of a fifth peak *E*, not shown in the figure, which occurred between fractions 145 and 160, were then analysed by paper chromatography both before and after hydrolysis (Table 3). Fraction *A*, which gave no colour with ninhydrin before hydrolysis, was MC-phenylalanine and, allowing for a correction factor based on the data in Table 2 (100/81·7), it represented

Table 3. *Chromatographic analysis of methoxycarbonyl-insulin hydrolysate*

See also Fig. 2.

Peak	Amino acids identified: Before hydrolysis	Amino acids identified: After hydrolysis	Groups by titration/mole (5732 g.)
A	None	Phenylalanine	0·82
B	None	None	—
C	None	Phenylalanine + + +	—
		Valine + + +	—
		Aspartic acid + + +	—
		Glutamic acid + +	—
D	None	Glycine	0·77
E	Phenylalanine + + + +	—	—

0·82 group of *N*-terminal phenylalanine/mole. Fraction *B* was small, and gave no detectable colour with ninhydrin either before or after hydrolysis. Fraction *C* gave no colour before hydrolysis and was probably a mixture of MC-Phe. Val. Glu. Asp and MC-Phe. Val. Glu., peptides whose occurrence might be expected from the work of Sanger (1949). Allowing for three titratable groups, the amount present was 0·16/mole, which would raise the value for *N*-terminal phenylalanine to 0·98 group/mole. Fraction *D* was MC-glycine, representing 0·77 group of *N*-terminal glycine/mole. Fraction *E* gave a strong spot for phenylalanine before hydrolysis and heralded the emergence of unmodified amino acids and peptides.

β-Lactoglobulin. Chromatographic analysis (Fig. 3) showed main peak *A* for MC-leucine, equivalent (corrected) to 1·81 groups of *N*-terminal leucine/mole. This was followed by three small peaks, *B*, *C* and *D*, where the fractions represented appeared to consist of MC-peptides in which residues of leucine (especially) and valine predominate (Table 4). It is known that a peptide bond between these two residues is hydrolysed only with difficulty. While there is no direct evidence that MC-leucyl-peptides are present, the inference is strong and we conclude that the number of *N*-terminal leucine residues is more likely to be three, in agreement with Porter (1948), than two, as given by Fraenkel-Conrat (1956). All the fractions embraced in peak *E* gave an immediate reaction with ninhydrin and must therefore have contained peptides or free amino acids.

As the material of peaks *C* and *D* might also have contained small amounts of acidic peptides an attempt was made to remove these before elution analysis in the following way (cf. Paulson & Deatherage, 1953, 1955). A fraction of the hydrolysate was treated at the boil under reflux with Dowex-50 in the acid form [protein (mg.): resin (g.): 0·05 N-HCl (ml.); 1:30:500] for 24 hr. It was expected that all peptides bearing a free amino group, and any amino acids, would be adsorbed and that MC-amino acid or peptide would remain in solution. After removal of the resin by filtration, and the excess of HCl by evaporation in the usual way, the product was submitted to elution analysis. The main peak *A* was again due to MC-leucine, but equivalent to only 1·66 groups of *N*-terminal leucine/mole, showing a small but significant destruction of the MC-amino acid. Peaks *B* and *C* were smaller than before, showing that the material represented had also undergone partial destruction. Peaks *D* and *E* were no longer apparent, but a new peak appeared about midway between where they would have occurred. This was shown to be due to pyrrolidonecarboxylic acid, arising from the employment of too high a temperature in the pretreatment of the material before transfer to the column. The loss of MC-leucine suggests that treatment at the boil with Dowex-50 under these conditions is inadvisible.

Rabbit tropomyosin. Chromatographic analysis of the hydrolysate gave one main, yet small, peak moving slightly ahead of where MC-leucine would be expected to emerge, followed by a succession of even smaller peaks. The fractions representing the main peak gave, after hydrolysis, a strong spot for leucine and weaker spots for aspartic and glutamic acids. The material was probably acidic MC-peptides or peptides, and to remove the latter we varied the treatment with Dowex-50 in light of the experience with lactoglobulin mentioned above. A portion of the hydrolysate (2 ml.) was evaporated to dryness *in vacuo*. The residue was dissolved in 0·05 N-HCl, Dowex-50 in the acid form was added [protein (mg.): resin (g.): 0·05 N-HCl (mg.); 1:30:100] and the mixture stirred at room temperature for 3 hr. It was then filtered, the resin washed, and the collected liquors were evaporated to dryness *in vacuo*. The residue material was then transferred to the Celite column in the usual way. Chromatographic analysis (Fig. 4) gave a succession of peaks, but the titration equivalent for each of

Table 4. *Chromatographic analysis of methoxycarbonyl-β-lactoglobulin hydrolysate*

See also Fig. 3.

Peak	Amino acids identified: Before hydrolysis	Amino acids identified: After hydrolysis	Groups by titration/mole (37 000 g.)
A	None	Leucine	1·81
B	None	Leucine + + +	(0·2–0·3)
		Valine + + +	—
C	None	Glutamic acid +	(0·3–0·9)
		Valine + + +	—
		Leucine + + + +	—
D	None	Leucine + +	—
E	Peptides + + + +	Glutamic acid + +	—
		Leucine + + + +	—

Table 5. *Chromatographic analysis of methoxycarbonyl-tropomyosin and methoxycarbonyl-ovalbumin hydrolysates*

Tropomyosin, mol.wt. 53 000: peaks *A–D*: Ovalbumin, mol.wt. 45 000: peaks *E–H*. See also Figs. 4 and 5.

	Amino acids identified		
Peak	Before hydrolysis	After hydrolysis	Groups by titration/mole
A	—	—	0·37
B	—	—	0·15
C	—	Glutamic acid + + + Glycine + + + + Lysine + Leucine + + + +	0·15–0·3 — — —
D	None	Glycine + + + + Lysine + Leucine + + Alanine trace	— — — —
E	—	Glutamic acid Lysine + Valine +	0·1–0·2 — —
F	—	—	0·18
G	—	—	0·05
H	—	Glutamic acid + + Aspartic acid + Serine + + Glycine + + Lysine + Valine + + +	— — — — — —

these, except *D*, represented far less than 1 group/mole (Table 5). As the amount of material was small, an amino acid analysis representing peaks *A* and *B* was not possible. Peaks *C* and *D* showed the presence of complex peptides. The new procedure, within the limits that it is at present being used, would thus appear to confirm Bailey's (1953) original finding that this protein has no *N*-terminal residue.

Ovalbumin. The hydrolysate was treated with Dowex-50 at room temperature in the same way as that of tropomyosin. Chromatographic analysis (Fig. 5, Table 5) did not reveal any *N*-terminal residue, confirming earlier work in which FDNB had been used (cf. Fraenkel-Conrat, 1956).

DISCUSSION

The present investigation has admittedly dealt with the stability and chromatographic behaviour of only a few of the possible MC-amino acids that could be present in the hydrolysate of an MC-protein, but even so it would appear that the procedure for the determination of the *N*-terminal residues in proteins which has been evolved from these exploratory studies has given results of value. Until similar data can be obtained for the other amino acids, especially those with a second potentially reactive group, the information that can be deduced from the present chromatographic analyses must be regarded as provisional only and the results as subject to revision. The analysis of MC-insulin nevertheless was in good accord with the known structure of this protein. Of the other three proteins investigated the results are in agreement with those obtained by the DNP procedure. On present evidence therefore the new MC procedure has few, if any, features that are superior in practice to those of the well-established procedure of Sanger (1945). Steric hindrance, if indeed it is a factor in the case of tropomyosin or ovalbumin, is no less effective when the molecular size of the reacting group is reduced. The possibility remains, of course, that failure to react with some or all of the *N*-terminal residues in a protein may be due to the charge distribution in the vicinity of the amino group concerned, as has been recently discussed by McPhee (1956) in a different context dealing with the reactivity of disulphides.

SUMMARY

1. The procedure of Sanger (1945), which employs 1-fluoro-2:4-dinitrobenzene, fails to reveal the presence of any *N*-terminal residue in proteins such as tropomyosin and ovalbumin.

2. As an alternative condensing agent, methoxycarbonyl chloride, of lower molecular weight and hence perhaps less prone to steric factors, has been investigated.

3. Methoxycarbonylamino acids are shown to be fairly stable in 8·6N-hydrochloric acid at 37°.

4. During condensation with methoxycarbonyl chloride proteins remain soluble in the medium, thereby aiding the attainment of full substitution.

5. The methoxycarbonylamino acids given on subsequent hydrolysis of the condensed protein in 8·6N-hydrochloric acid at 37°, being strongly acidic, can be readily separated from the other products of hydrolysis on a buffered column of Celite. A good resolution is obtained by using gradient elution.

6. The procedure has been applied to insulin, β-lactoglobulin, rabbit tropomyosin and ovalbumin. The results confirm those obtained by the dinitrophenyl procedure and do not suggest that methoxycarbonyl chloride has any particular advantage over 1-fluoro-2:4-dinitrobenzene as an agent for the determination of *N*-terminal residues in proteins.

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An Assay of Insulin by Fibril Formation from Small Samples of Pancreas

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A paper-chromatographic technique for the isolation and assay of insulin from 1 g. samples of foetal ox pancreas has been described in an earlier report (Grodsky & Tarver, 1956). It was effective at levels of insulin above 100 μg./g. of tissue, but attempts to increase the sensitivity so as to make it applicable to studies of insulin metabolism, where the hormone level would be very much lower than this, have been consistently marred by the presence of relatively large amounts of other protein material which moved from the origin at the same rate as the insulin itself. For this reason it has been thought worth while to investigate an alternative procedure.

Waugh (1946), following the pioneer observation of Du Vigneaux, Geiling & Eddy (1928), observed that insulin in acid solution separated as fibrils when the temperature was raised. A preparation of these insoluble fibrils would act as a seed in crude dilute solutions of insulin, and when this was carried out at the right pH the insulin was quantitatively precipitated.

This method of assay has been criticized by Foster, MacDonald & Smart (1951) and by Light & Simpson (1956), who have reported that recoveries may be erratic in the presence of a crude mixture of tissue proteins. There is, indeed, evidence that globin (Foster *et al.* 1951), muscle protein, serum albumin and fibrin (Waugh, Wilhelmson, Commerford & Sackler, 1953) can form insoluble aggregates under the prescribed conditions, and Sutherland, Cori, Haynes & Olsen (1949) found that glucagon could be precipitated as part of the fibril mat.

By suitable modification of the Waugh (1946) procedure it has been found possible to assay the insulin in 1 g. of adult animal pancreas without any apparent interference from glucagon or other extraneous proteins. It has been applied under limited conditions to the pancreas of several animal species and the efficiencies of seed fibrils from pig, sheep and ox insulins have been compared.

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EXPERIMENTAL

Materials. Crystalline ox insulin was obtained from Boots Pure Drug Co. Ltd., Nottingham (batch no. 9011G). The sterile solution of glucagon was prepared by Eli Lilly and Co., Indianapolis, Ind., U.S.A. (batch no. 208–158B 197). I am grateful to Doctors F. Sanger, D. S. H. W. Nickols and L. F. Smith for samples of esterified insulin, of insulin that had been treated with carboxypeptidase so as to remove the terminal alanine and asparagine residues, and of the *A* and *B* fractions of oxidized insulin. The bovine serum albumin was obtained from Armour and Co. Chicago, Ill., U.S.A.

Assay of insulin by paper chromatography. The procedure of Grodsky & Tarver (1956), with minor changes dictated partly by the atmospheric conditions of the laboratory and the filter-paper sheet available, was first explored. If the insulin band, after staining with bromophenol blue, was extracted with 0·1 M-K_2HPO_4 instead of with acidic ethanol and the total volume of eluting solution was reduced from 25 to 6 ml., an eightfold increase in sensitivity of the colorimetric determination was thereby obtained. Nevertheless, when this modified technique was applied to extracts from liver tissue, which contain an appropriate mixture of proteins other than insulin, a small amount of foreign material, approx. 20–25 μg./g. of tissue, travelled at the same rate as insulin itself. Other products which interfere in the same way were glucagon (Grodsky & Tarver, 1956), esterified insulin, insulin modified with carboxypeptidase and insulins from sheep and pig, all of which move at the same rate as ox insulin. The procedure was clearly not applicable at the level of insulin dealt with in this paper and was accordingly abandoned.

Assay of insulin by precipitation of fibrils. The pancreatic tissue was treated by the technique of Grodsky & Tarver (1956) until the stage when the crude insulin was precipitated with alcohol–ether. When this precipitate was extracted with 0·1 N-HCl (0·5 ml.) containing 0·1 % NH_4Cl, the major part of the protein material passed into solution. The extract was filtered through a fluted Whatman no. 2 filter paper into a flat-bottomed vial of the type described below. A second extract was prepared and used to wash the filter paper. The filtrate, which should be clear and at approx. pH 1·5, was adjusted to 1 ml. by adding water. Standard solutions of insulin of the same pH were prepared by dissolving the crystalline material in 1 ml. of 0·05 N-HCl containing 0·1 % NH_4Cl.

After preparation of the clear solution, 20 μg. of freshly prepared insulin seeding fibrils (Waugh, Thompson & Weimer, 1950) were added to each vial, the magnetic-stirrer rods ('fleas', see below) were inserted and the vials firmly sealed with close-fitting rubber stoppers. Fig. 1 shows the apparatus used for stirring the contents of the vials at a uniform rate. A glass-walled constant-temperature water bath with stirrer was placed on two identical magnetic stirrers (Towers Co. Ltd., Manchester). The radius of sweep of the electromagnet in these instruments is 4·5 cm. Nine holes (outside diam. 1·2 cm.) each large enough to accommodate loosely a vial were drilled in a circle 4·5 cm. from the centre of each of two stainless-steel disks. The centre of each disk was drilled and tapped to fit a stainless-steel rod which acted as a handle. Alongside each hole was fastened a small strip of spring metal which held each vial firmly in place. The acid-resistant nickel–silver rods ('fleas') which rotate freely in the moving electromagnetic field, were approx. 7 mm. long and of 2 mm. diam. By placing the holders over the centre of the magnetic stirrer, the 'fleas' in the vials lying on the path of the electromagnet could be agitated. By adjusting the speed of the stirrer, this agitation could be transformed into a rotary motion which caused a rapid stirring of the contents of the vials with little or no surface foaming.

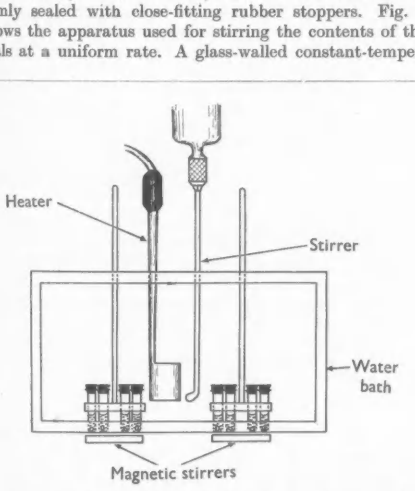

Fig. 1. Apparatus for stirring the vials used for preparing insulin fibrils.

The seeded solutions were stirred at 45° for 16–18 hr. The contents of the vials were then transferred to 12 ml. centrifuge tubes. Each vial was washed with 0·05 N-HCl (1 ml.) containing 0·1 % NH_4Cl, and any fibrils adhering to the glass surface of the vial were removed with a glass rod. The fibril suspension and wash were combined, the fibrils centrifuged down and the supernatant discarded. The fibrils were washed once in the acid solution and twice in 1·5 ml. of methanol.

Two methods for the quantitative determination of the insulin were used. After the last methanol wash, the fibrils were re-suspended in 1·5 ml. of the acid solution. The extinction of this suspension at 275 mμ was compared with those obtained from similarly treated standard solutions of insulin. Alternatively, the fibrils were washed once more with the acid solution, dissolved by boiling in 1 ml. of NaOH for 15 min., the solution was centrifuged to remove insoluble particles, the volume adjusted with water and the tyrosine content of the solution compared with similarly treated solutions of insulin, the extinction being measured at 293 mμ (Beaven & Holiday, 1951).

Comments. Waugh *et al.* (1950) were able to produce insulin fibrils from commercial 'still concentrates' dissolved in H_2SO_4. In the present investigation the crude mixture of proteins from the alcoholic extracts of pancreas was not readily soluble in 0·1 N-H_2SO_4. Filtrates were cloudy and during fibril formation a large amount of non-specific material separated. The addition of 1 % Na_2SO_4 increased the amount of extraneous precipitation. The protein mixture, however, was readily soluble in 0·1 N-HCl containing 0·1 % NH_4Cl. The final washing of the separated fibrils with methanol removed a large amount of extraneous material, and good recoveries of added insulin could not be obtained if this step were omitted. Although the stirring technique resulted in a minimum of splashing, the fibrils massed in a ring at the surface of the liquid on the sides of the glass vials. This partial separation of the fibrils, however, did not affect the assay.

The two methods used for estimating the insulin fibrils gave closely agreeing results. That based on

a direct reading was preferred, as it called for less manipulation. The standard error obtained by repeated assays of the same crude insulin preparation was ±5%.

Specificity of the method. Waugh *et al.* (1953) reported that many proteins form fibrils and, of those investigated, albumin is normally present in pancreas and could be expected to interfere with the assay. In the present procedure, however, the major portion of albumin was precipitated during the alkaline-alcohol treatment for preparation of the crude insulin. Glucagon could also be a possible contaminant (Sutherland *et al.* 1949). Neither albumin nor glucagon forms fibrils under the conditions of the present procedure, nor do they interfere with the fibril formation of insulin whether in pure solution or in extracts of pancreas.

RESULTS

Fig. 2 demonstrates the recovery of insulin which had been added to the protein precipitated from acid alcoholic extracts of rat liver and ox pancreas. Recoveries of increasing amounts of added insulin (including that added as seeding fibrils) are linear and 95% quantitative.

To investigate the possibility of seed specificity, as described by Waugh *et al.* (1950), standard solutions of ox, pig, sheep and calf insulins, carboxypeptidase-modified insulin, esterified insulin and Sanger's *A* and *B* fractions of oxidized insulin were seeded with fibrils from ox, pig and sheep respectively. Although more than 250 observations were made, the recoveries did not demonstrate any consistent species difference in the fibrils used (Table 1). Fibril formation occurs in the absence of seed, but generally in low yield (Table 1).

Values for the insulin content of pancreas obtained from samples of various animal species are given in Table 2. The figures for standard error refer to the individual values and because the number of animals available for study was limited,

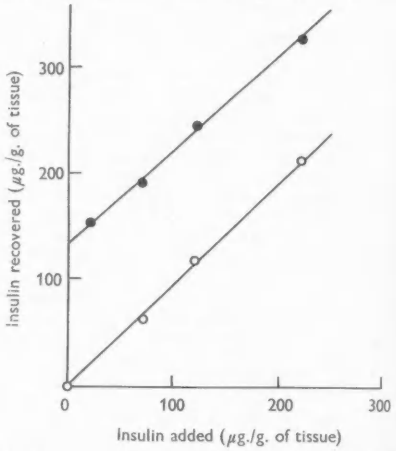

Fig. 2. Recovery of insulin added to the alcohol–ether precipitate obtained from the acid–ethanol extracts of: ●, ox pancreas; ○, rat liver.

Table 1. *Recovery of insulin from pure solution by the use of fibrils from various species as seed*

Solutions contained 200 μg. of modified or of unmodified insulin; recoveries are expressed as percentages. Standard error, 4·9%.

Seed fibrils	Species of insulin				Oxidized insulin		Esterified insulin	Decarboxylase-modified insulin
	Ox	Pig	Sheep	Calf	*A* fraction	*B* fraction		
Ox	103·6	103·1	100·8	99·2	0·0	100·1	91·0	110·3
Pig	106·0	97·3	99·9	98·0	—	—	—	—
Sheep	108·7	93·0	97·1	101·5	—	—	—	—
None	80·5	93·3	86·6	97·1	—	43·7	—	—

Table 2. *Yield of insulin from pancreas of various species*

Results are expressed as μg./g. of pancreas; standard error, 5·2%. Figures in parentheses refer to the number of pancreases pooled.

Seed fibrils	Pancreas				
	Ox (5)	Pig (1)	Calf (2)	Cat (3)	Rat (48)
Ox	140	143	485	56	41
Pig	—	119	400	—	41
Sheep	—	125	460	56	31
None	—	151	436	—	19
Quoted by Haist (1944)	150–170	100–150	500	35–125	30–65

no attempt has been made to determine the statistical average of insulin content for each species. No consistent differences were observed when seed from different species was used.

DISCUSSION

The observation that the use of sulphuric acid in the original fibril method described by Waugh (1946) caused considerable precipitation of foreign protein could explain the lack of specificity reported by Foster *et al.* (1951) and Sutherland *et al.* (1949). Most of this interference was prevented by using hydrochloric acid and rapid stirring. Glucagon did not form fibrils. Small amounts which might come down with the insulin (Sutherland *et al.* 1949), although possibly of high biological activity, were negligible as protein impurities.

Contrary to Waugh *et al.* (1950) and Foster *et al.* (1951), who found pig fibrils to be the most efficient seed for recovery of insulin, no consistent species difference was observed. It may be suggested that the long period of rapid stirring used in the present procedure carried the fibril formation to completion so that individual differences in rate were unobservable.

The formation of fibrils by modified insulins agrees with the earlier observations of Waugh *et al.* (1950), of Koltun, Waugh & Bear (1954) and of Mommaerts & Neurath (1950). Evidence that the *B* fraction of oxidized insulin also forms fibrils suggests that the disulphide bonds are not involved and supports the view of Koltun *et al.* (1954) that the linkage may be due to the interaction of small non-polar side-chain groups.

Values for the insulin content of pancreas obtained from samples of various animal species (Table 2) are in general agreement with those quoted in the review by Haist (1944).

The present procedure, though perhaps not specific for the unmodified insulin molecule, appears to give satisfactory assays at the insulin level normally present in adult-animal pancreas. It thus seems to be a suitable one for metabolic studies which may be concerned with the level of insulin during the incubation of pancreatic slices, one of the purposes for which it was designed.

SUMMARY

1. A chemical method for the assay of insulin in 1 g. samples of pancreas tissue, utilizing the phenomenon of fibril formation, is described.

2. Recoveries are linear and near quantitative. Although some modified insulins can form fibrils, a general protein mixture from liver, as well as albumin and glucagon, does not interfere with the assay.

3. The levels of insulin found in the pancreas of ox, pig, sheep, calf, cat and rat were comparable with those obtained by biological-assay methods.

4. No differences in insulin yield were observed when seeding fibrils from ox, pig, and sheep insulins were used.

5. An apparatus is described for the simultaneous determination of 18 samples.

I should like to thank Dr A. C. Chibnall for suggestions. The continued interest, advice, and helpful discussions of Mr Maurice Rees are gratefully acknowledged.

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The Use of [^{14}C]Amino Acids to Study Sites and Rates of Antibody Synthesis in Living Hyperimmune Rabbits

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Evidence for antibody production in various organs and tissues of immunized animals has been obtained in the main by three different methods. The first used (e.g. Pfeiffer & Marx, 1898) was to show that antibody could be found in particular tissues in higher concentrations than in the blood plasma at the same time. The theoretical objections to this method, namely, that high concentrations of antibody might occur by some process of local accumulation rather than of synthesis, have been met by Oakley's comparison of the responses to two different antigens injected at separate sites (McMaster & Hudack, 1935; Ehrich & Harris, 1942; Oakley, Warrack & Batty, 1949).

The second method has been to show that tissues from an immunized animal can produce antibodies either *in vitro* (e.g. Fagraeus, 1948; Askonas & White, 1956; Stavitsky, 1955) or after transplantation to another host (e.g. Topley, 1930; Oakley, Batty & Warrack, 1951; Sterzl, 1955).

The third has been the elegant fluorescent-staining technique of Coons, whereby antibody-containing cells can be directly demonstrated in histological preparations (Coons, Leduc & Connolly, 1955).

Although many antigens have been used, administered to different species of animal by different routes and for varying periods of time, results obtained by the three methods agree in pointing to the spleen, lymph nodes, bone marrow and (sometimes) the tissues at the sites of injection as being those chiefly involved in antibody synthesis. Most of the studies have been concerned with establishing the importance of a tissue or a particular type of cell in such synthesis.

We were interested to see whether it might be possible to determine the actual rates and amounts of antibody production by different tissues in living immunized animals, and to get some idea of the time required for synthesis of antibody molecules and for their appearance in the blood stream. Although studies on hyperimmune rabbits are of limited interest only, such questions are of relevance to the general problem of protein metabolism in higher animals; and antibodies, because of the ease with which they can be isolated specifically in small amounts from complex mixtures, provide unique material for such a study.

General plan of the work. Rabbits were immunized by intravenous injections of killed pneumococci type 3 over periods of several weeks, until the serum-antibody level was high and, as nearly as possible, steady. Under these conditions, the overall rates of production and degradation of antibody are constant and equal, and we assumed that each antibody-producing tissue was synthesizing and liberating antibody into the circulation at a steady rate. A mixture of uniformly labelled [^{14}C]amino acids was then administered intravenously to a series of rabbits, and at various time intervals thereafter the animals were killed, bled and rapidly perfused with Ringer solution. A mixture of uniformly labelled amino acids was purposely chosen in the hope that variations in behaviour of individual amino acids would be evened out.

The antibody was extracted from different tissues, isolated by specific precipitation, and its specific ^{14}C-radioactivity compared with that of the plasma antibody, and of the free amino acid pool in the plasma and the tissues. Owing to the uncertainty introduced by the fact that tissue antibody is derived partly from intracellular antibody and partly from trapped lymph, it was necessary to be able to correct for the lymph contribution. This was done by administering, several days beforehand, a quantity of antibody of *the same kind* which had been labelled with ^{131}I. The ^{131}I-labelled antibody mixes with circulating antibody, becomes distributed in the same way between plasma and lymph, and is broken down at the same rate (Humphrey & McFarlane, 1954; Cohen, Holloway, Matthews & McFarlane, 1956). The ^{131}I-labelled breakdown products are promptly excreted, and there is never any appreciable quantity of ^{131}I-labelled intracellular antibody. Hence by measuring the ^{131}I-activities of the antibody recovered from tissue extracts, and of the plasma antibody obtained at the same time, an accurate estimate of the extracellular antibody could be made.

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Corrections, which are described below, had also to be made for completeness of recovery of antibody, and for contamination with other materials. Furthermore, since we attempt to relate the specific radioactivity of intracellular antibody to that of the plasma free amino acids, it was necessary to determine the relationship between the latter and the free amino acids of the tissues. Finally, since other non-protein materials besides amino acids become labelled, we had to investigate to what extent the non-protein radioactivity of plasma was derived from the injected amino acids and to what extent from their metabolic products.

The results have been used to attempt to calculate rates of synthesis of antibody in different tissues of the whole animal. Because of the numerous corrections and assumptions involved, the calculations are only very approximate. Their significance is assessed in the discussion.

MATERIALS AND METHODS

Animals. Adult white or sandy lop rabbits were used. They were bred at the National Institute for Medical Research, London, and were maintained on pelleted diet no. 18 (Bruce & Parkes, 1940), supplemented with hay.

Antigens. Formalin-killed type 3 pneumococci, prepared according to Kauffman, Björneboe & Vammen (1938), were used for immunization. Type 3 capsular polysaccharide, prepared according to Heidelberger, Kendall & Scherp (1936), was used in precipitation tests

Immunization of rabbits. Pneumococci were injected intravenously, at 2- to 3-day intervals, in one or more courses of about 1 month, until a level of at least 5 mg. of antibody/ml. of serum was obtained. The injections were continued until 1 or 2 days before the experiment, to ensure that antibody production was maintained at a steady rate. Unpublished results of experiments showed that the rate of production of antibody against capsular polysaccharide began to fall off within about 4 days of the last intravenous injection of pneumococci. At least 90% of the precipitating antibody produced is directed against the capsular polysaccharide.

Procedures for immunization and isolation of antibody

Two or more rabbits were used in each experiment, except in the preliminary work, in which only one rabbit was used. Sodium iodide was added to the drinking water (50 mg./l.) 2–4 days before the experiment in order to dilute inorganic ^{131}I, liberated by breakdown of the labelled protein, and so to minimize ^{131}I-uptake by the thyroid. Rabbit serum, containing anti-pneumococci type 3 antibody, or a purified pneumococci type 3 antibody fraction, was labelled with ^{131}I by the rapid mixing method of McFarlane (1956). Into the ear vein of each rabbit, 250–500 μc of ^{131}I-labelled anti-pneumococci type 3 antibody was injected. The specific activity was approx. 10 μc/mg. of protein. Blood samples were taken from the ear daily, or at other suitable intervals, until the experiment was completed.

Two to 4 days after the injection of the ^{131}I-labelled antibody, the rabbits were injected intravenously with an acid hydrolysate of approx. 25 μc (0·5 mg.) of uniformly ^{14}C-labelled *Chlorella* protein, obtained from the Radiochemical Centre, Amersham (Catch, 1954). After hydrolysis and removal of HCl about 21 μc of soluble material remained. A sample of blood was taken 3 min. later. Serum or plasma was separated at 2° and used for the estimation of radioactivity of ^{14}C-labelled amino acids in the plasma. After a chosen interval of time the rabbit was again bled from the ear, injected with 5 mg. of heparin intravenously and anaesthetized with Nembutal. The thorax was immediately opened, the right auricle was removed and 2–3 l. of Ringer solution was perfused via the left auricle, the heart being used as a manual pump. This method resulted in a very efficient removal of the blood, but some tissue fluid was inevitably left behind.

These tissues were then removed and placed in ice: lung, bone marrow (obtained from the long bones), liver, spleen, appendix, lymph nodes (cervical, axillary, inguinal, mesenteric, popliteal). The tissues were cleaned from adhering fat, rinsed with cold tap-water, blotted, weighed and homogenized at 0° in an Ato-Mix homogenizer (Measuring and Scientific Equipment Ltd., London), enough fluid being added to make a workable suspension. The fluid consisted of 0·9% NaCl solution buffered with 0·015 M-sodium phosphate, pH 7, containing 1:10 000 merthiolate. A 5% solution of γ-globulin prepared from normal rabbits was then added in the proportion of 10–40 mg./g. wet wt. of tissue, with the purpose of limiting adsorption of antibody by the tissue fragments. The total volume of suspension at this stage was 3–15 times the volume of tissue taken.

Extraction of antibody. The antibody was extracted by freezing the suspensions at −10°, thawing and centrifuging at 2°, at 3000 rev./min. for 30 min. This procedure was repeated three times, with diminishing volumes of buffer for extraction. Between the first and second freezing the macerated tissues were ground in a mortar to ensure complete destruction of the cells.

The three extracts were pooled and centrifuged in a high-speed angle centrifuge, at 12 000 rev./min. for 3–6 hr. in the cold, until absolutely clear. They were left overnight at 2°. If clear, antibody was precipitated as described below; if not, they were centrifuged again and left to stand for a further period. This was done in order to ensure that non-specific precipitation would be avoided as far as possible.

Antibody precipitation. To the clear extracts was added a concentrated neutralized acid hydrolysate of serum proteins (120 mg. of amino acids and 72 mg. of NaCl/ml.), sufficient to give a final concentration of 20 mg. of amino acid/ml. This concentration did not interfere with specific precipitation, and almost completely prevented adsorption of radioactive free amino acids. The extracts were centrifuged after standing overnight (3000 rev./min. for 30 min.). The amounts of antibody available were too small to allow accurate predetermination of the amount of antigen required for each extract. Antibody was therefore precipitated by stepwise addition of small amounts of antigen until no further precipitation occurred. Since precipitation in the antigen–antibody system in question is not inhibited by even a two- to three-fold excess of antigen, this procedure is unlikely to result in loss of antibody. After the last addition of antigen the extracts were left overnight

at 2° to complete the deposition of antibody, which was always somewhat fibrous. If certain extracts were left in the cold for longer periods, non-specific precipitates, recognizable by their different consistency, were sometimes formed. On the few occasions when, in spite of these precautions the precipitates were obviously contaminated with non-specific material, this was noted. No precautions were taken to remove complement.

Estimation of antibody. The antibody precipitates were washed twice in saline with 0·9% NaCl, followed by 50% (v/v) aq. methanol, methanol and finally ether; they were then dried and weighed. Alternatively, the precipitates were dissolved in 0·1N-NaOH and the antibody was estimated by measurement of the optical density at 280 mμ.

The precipitates contained 5% by wt. of capsular polysaccharide. Since this was often within the weighing error of the small amounts of antibody which we were handling, the contribution of the antigen was neglected throughout.

Specific radioactivity of free amino acids in serum and tissues

We did not attempt to isolate each amino acid individually, but only to estimate the average specific activity of the carbon in the free amino acid mixtures present in the samples. Since the technical problems involved were different for serum and for tissues the procedures used are described separately.

Plasma-free amino acids. Two methods of estimation were used: In the first method (referred to as 'glycine dilution'), serum samples, usually fresh but occasionally after storage at −10° for a few days, were dialysed in cellophan sacs against three changes of equal volumes of distilled water. The diffusates were pooled, and the amino N in a measured fraction was measured by the naphthoquinone method of Russell (1944). The remainders of the diffusates were concentrated to a small volume by evaporation at 105°, and to them was added sufficient glycine to dilute the amino acid C suitably for subsequent measurement of specific radioactivity. In order to make the dilutions, a factor had to be assumed for converting amino N into amino acid C. The factor depends upon the composition of the amino acid mixture present in the serum, which may vary from sample to sample. We used the figure of amino N × 4·4 for reasons given below.

The second method (referred to as 'ninhydrin-CO_2') is based on the following considerations. Since the ^{14}C-labelling of the amino acids administered was uniform, the specific activity of the terminal carboxyl C should be identical with that of the total C. Furthermore, the terminal carboxyl C can be specifically liberated from free α-amino acids by treatment with ninhydrin, and possible interference by other ^{14}C-labelled metabolites is thereby eliminated.

Provided that the average carbon content of labelled amino acids in rabbit plasma is close to the average for the algal-protein hydrolysate, which is true if calculations are based on the values given by Duchateau & Florkin (1954) for the composition of free amino acids in rabbit plasma, measurement of the specific activity of the terminal carboxyl C seemed likely to give the best estimate of the specific ^{14}C-activity of the free amino acids. The disadvantage of the method was that large plasma samples were required to yield sufficient CO_2 for estimation and counting. In certain instances, however, this method was used, 15–25 ml. of plasma being mixed with an equal volume of 10% (w/v) trichloroacetic acid, and treated in the manner outlined for the tissue extracts below.

Tissue-free amino acids. Since the cellular make-up of tissues of hyperimmunized rabbits may differ from normal, the animals were first subjected to one or more courses of pneumococci type 3 injections, so as to make them comparable with the animals used for antibody studies. Algal [^{14}C]protein hydrolysate was injected intravenously, and after a suitable time interval the rabbits were anaesthetized and immediately perfused via the left auricle with about 1 l. of ice-cold saline until the returning fluid was almost free from red cells. This took 2 min. The organs were removed as rapidly as possible and placed in crushed ice, after which they were cleaned, weighed and dropped into liquid air. In order to obtain bone marrow, the long bones were freed as far as possible from muscle before immersing in ice; the bones were removed one at a time, the ends were cut off and the marrow expelled cleanly by applying compressed air to one end. The time between the end of the perfusion and immersion in liquid air was 2–3 min., except for bone marrow, which took 5–8 min.

The frozen tissues were crushed and then ground with a volume in millilitres of 10% (w/v) trichloroacetic acid equal to their weights in grams. The resulting fine suspensions were centrifuged hard, and the supernatant fluid was extracted thrice with peroxide-free ether in order to remove trichloroacetic acid. The volume was reduced to 1–2 ml. by evaporation at 105° and, after filtration to remove insoluble material, the tissue extract was treated with ninhydrin at pH 2·5, and the free amino acid carboxyl CO_2 collected in baryta by the technique of Van Slyke, McFadyen & Hamilton (1941). In this technique it was necessary to use silicone antifoam (MS Antifoam A, Hopkin and Williams, Ltd., Chadwell Heath, Essex), since it became apparent in our earlier experiments that other antifoaming agents are liable to get into the counting tube and to cause quenching. The $BaCO_3$ precipitate was transferred to one arm of a Rittenberg tube and an approximately equal volume of conc. H_2SO_4 was placed in the other arm (H_2SO_4 was used instead of HCl in order to avoid getting HCl into the gas line). The CO_2 was liberated and its specific activity was measured (see below). Liberation of CO_2 was usually incomplete, and when quantitative recovery was required the $BaCO_3$ was first washed thrice with CO_2-free water, dried at 105° and weighed.

Although this method measured free amino acids present in the lymph, besides those inside the cells, the independent measurements of contamination by lymph, made by means of [^{131}I]proteins, showed that extracellular amino acids could not have made up more than 5% of the total.

Measurement of radioactivity. ^{131}I was measured by counting in a well-type scintillation counter of conventional design. The material to be counted was either in the form of a small dry pellet or dissolved in 3 ml. of dilute NaOH.

Specific ^{14}C-activities were measured after combustion to CO_2 by the method of Bradley, Holloway & McFarlane (1954). For measurements of specific activities of plasma free amino acids the materials were diluted with known amounts of carbon, added as glycine, sufficient to give a suitable volume of gas in the counter. In those instances

where the activity of terminal carboxyl carbon only was measured, no dilution was made (the amounts of radioactivity injected having been chosen so as to yield samples with activities within the capacity of the counting equipment).

For greater ease of presentation the results are presented in two parts, the first dealing with antibody recovery and radioactivity, and the second with free amino acids in plasma and tissues. They are, however, considered together in the Discussion.

RESULTS OF ANTIBODY STUDIES

Reliability of methods

Recovery of antibody from tissues. Since the amounts of antibody present in the tissues were small, some losses were inevitable. Non-specific adsorption of antibody on to tissue debris was minimized by the addition of a large excess of normal rabbit γ-globulin. Significant binding by intracellular antigen is unlikely to have occurred, because the amounts of antigen injected were relatively minute and because antigen was not detectable in control material stained by the fluorescent-antibody technique of Coons & Kaplan (1950). Nevertheless, it was important to know how efficient were the antibody recoveries. Although these could not be measured directly, an estimate was obtained by adding to tissues of normal rabbits known amounts of ^{131}I-labelled antibody against capsular polysaccharide, in quantities comparable with those found in an average experiment, and attempting to recover the antibody in the usual way. Since the specific ^{131}I-activity of the added antibody was known, measurement of the specific activity of the recovered precipitate made it possible to estimate the extent to which the recovered antibody was contaminated with inert material.

The procedure of stepwise addition of antigen involved warming the tissue extracts at 37° for a total of up to 2 hr. Since we feared that some destruction of antibody might occur during this period, the effect of heating lung and bone-marrow extracts for 6 hr. was also examined.

It will be seen from Table 1 that although the weights of precipitate recovered were usually little less than the weight of antibody added, there was a considerable admixture of non-antibody material which lowered the specific activity. This occurred especially when small amounts of antibody were present in large amounts of tissue (e.g. the appendix), so that the volumes of extract and the amounts of cell material were large. The result was to reduce the net recovery.

When bone marrow was incubated for 6 hr. the recovery was unchanged, but with lung it was 20% less, a reduction which was disregarded since the incubation period was at least three times longer than any used in practice.

In view of these results, it was clear that antibody extracted from tissues was liable to be contaminated with inert material and that extraction would be incomplete. Approximate corrections were therefore calculated, based on the mean values in the two control experiments, as shown in Table 1.

Radioactive contamination of recovered antibody. The following experiment was performed in order to assess the extent to which antibody recovered from an immunized rabbit, after administration of [^{14}C]amino acid, had become labelled by adsorption of amino acids or of highly labelled cell proteins. To the tissue extracts of two of the animals of our series were added known amounts of unlabelled anti-ovalbumin. These were specifically precipitated by addition of ovalbumin, before the antibody to capsular polysaccharide was recovered in the usual way. Specific ^{14}C-activities were

Table 1. *Recovery of antibody added to normal rabbit tissues*

The antibody added had been labelled with ^{131}I. For recovery procedures, see Methods. Net recovery = (wt. recovered × purity)/wt. added. Correction factors for losses during recovery from different tissues were calculated from the mean values of the corresponding percentages. Occasions when these factors were used are indicated in the text.

Tissue	Amount of antibody (mg.)		Specific activity of antibody (counts/min./mg.)		Purity of recovered antibody (%)	Net recovery (%)	Correction factor for	
	Added	Recovered	Added	Recovered			Purity	Wt. recovery
Lung	19·6	17·06	53 500	45 000	84	73	1·25	1·3
	10·8	7·25	16 100	12 300	76	51		
Bone marrow	9·8	8·8	51 600	36 000	70	63	1·60	1·15
	5·8	4·8	16 100	8 900	55	46		
Spleen	1·96	1·7	51 600	30 700	60	52	1·82	1·09
	1·0	1·0	16 100	8 090	50	50		
Appendix	1·96	1·2	51 600	30 000	58	36	2·20	1·71
	1·45	0·8	16 100	4 950	31	17		

measured, and those of the added anti-ovalbumin were only about 10% of those of the tissue debris and 1% of those of the antibody to capsular polysaccharide. Radioactivity due to non-specific adsorption was therefore insignificant.

As a further check, in another experiment the tissue extracts, after removal of antibody, were precipitated with trichloroacetic acid and the specific radioactivities both of the soluble non-antibody protein and of the insoluble tissue debris were measured. As will be seen in Table 2, the antibody radioactivities were about ten times greater than those of the remaining soluble material.

Correction for preformed extracellular antibody. Although most of the blood was removed by perfusion, there remained in the tissues an unknown amount of extracellular fluid whose antibody was inevitably mixed with any truly intracellular antibody present. This was allowed for in the following way. Two to 4 days before the animals were killed, 10–20 mg. of ^{131}I-labelled antibody to capsular polysaccharide (100–300 μc) was injected intravenously, and this became mixed with unlabelled antibody in the circulation. Since the ^{131}I-label is promptly lost once the labelled molecules become intracellular and are catabolized, and since antibody molecules remain intact only so long as they are extracellular (apart, of course, from those actually in the synthesizing cells) (Humphrey & McFarlane, 1954; Cohen *et al.* 1956), ^{131}I-labelled antibody could be used as a marker for extracellular antibody. About 3 days after injection of ^{131}I-labelled antibody, the specific ^{131}I-radioactivity of the antibody in the plasma is almost equal to that in the extracellular extravascular fluid. Thus if the specific ^{131}I-activity of the serum antibody at the time of death was A, and that of the antibody extracted from a tissue B, then suppose that there were x and y mg. of extra- and intra-cellular antibody respectively, then $B = xA/(x+y)$ counts/min./mg.; hence $x/(x+y) = B/A$, and $(B/A) \times 100$ is the percentage of the tissue antibody which was extracellular.

In some of our experiments, the plasma antibody had become labelled with ^{14}C to a significant extent during the time which elapsed after administration of [^{14}C]amino acids. Although the correction was small, it was made on the assumption that the ^{14}C label would be present in lymph antibody to the same extent as in the plasma (which is likely to be true for the very vascular tissues examined). Thus if a and b were the measured specific ^{14}C-activity of the serum antibody and tissue antibodies respectively, and p the calculated percentage of extracellular antibody in the tissue antibody (from the measurements of ^{131}I), true specific activity of tissue antibody $= (100b - pa)/(100 - p)$.

In Table 3 are given representative figures for the calculated percentages of extracellular antibody present in different tissues in various experiments. They are very variable for two reasons. One is that the efficiency of perfusion was by no means uniform, and the other that when the amounts of antibody were very small the error in weighing total antibody was such that it was impossible, for example, to distinguish between extracellular antibody contributions of 90 or 100%. Thus, in experiments with both ^{131}I and ^{14}C, it sometimes occurred that by measurement of ^{131}I all the antibody from a tissue appeared to be extracellular, and yet the antibody specific ^{14}C-activity was much higher than that of the serum antibody at the same time, indicating that part of the tissue antibody was actually intracellular. In such instances, however, no true estimate of the intracellular-antibody specific activity could be made, and the values were not used in subsequent calculations.

Table 2. *Distribution of radioactivity in tissue fractions*

Non-antibody material and antibody against capsular polysaccharide were isolated from the tissues of a rabbit 0·25 hr. after injection of 85 μc of [^{14}C]amino acids. The specific activities of the resulting fractions are expressed as counts/min./mg. of C.

Tissue	Antibody	Soluble non-antibody protein	Insoluble debris
Lung	6 750	699	926
Bone marrow	7 300	762	1318
Spleen	21 200	665	Not measured
Lymph gland	7 700	880	727
Appendix	7 900	608	900

Table 3. *Percentage of antibody found to be extracellular in tissue extracts after perfusion*

Three days before the rabbits were killed they were injected intravenously with 100–300 μc of ^{131}I-labelled antibody. Antibody was subsequently isolated from the perfused tissues shown and also from the serum. Comparison of the specific activities of the former with those of the latter enabled the percentages of antibody, persisting in extracts of the perfused tissues, to be determined. The amounts of ^{131}I-labelled antibody injected were sufficient to ensure that the measured radioactivities of the tissue antibodies were never less than 1½ times background, and usually very much higher.

Rabbit no.	Lung	Bone marrow	Spleen	Lymph glands	Appendix
28	32	74	24	71	100
24	30	100	91	63	17
2	45	44	15	54	34
19	38	25	8	50	35
5	63	65	35	83	71
23	56	41	10	66	No antibody

Table 4. *Antibody distribution in tissues of a rabbit immunized against two antigens*

The percentages of extracellular antibody in the perfused tissues were determined in a similar manner to that described in Table 3, except that a mixture of two antibodies labelled with ^{131}I had been injected 2 days previously. The mixture was obtained by iodination of 1 ml. of the animal's own serum with 270 μc of ^{131}I. At the time of death the serum contained (per ml.) 10·7 mg. of anti-ovalbumin and 6 mg. of antibody to capsular polysaccharide.

	Antibody extracted (mg.)		Extracellular antibody (%)	
Tissue	Anti-ovalbumin	Anti-polysaccharide	Anti-ovalbumin	Anti-polysaccharide
Lung	12·5	2·6	16·8	20·7
Bone marrow	12·1	3·5	69	74

Table 5. *Levels of antibody and ^{131}I-radioactivity in serum of rabbit in illustrative experiment*

About 20 mg. of antibody labelled with ^{131}I was injected intravenously, and blood samples were taken after 3 min., 24 hr., 48 hr. and 70 hr. The amount and radioactivity of the antibody in the serum was determined.

Date	Antibody (mg./ml.)	Specific ^{131}I activity (counts/min./mg.)
26. viii. 55	9·9	76 500
27. viii. 55	9·5	45 300
28. viii. 55	9·2	37 900
29. viii. 55 (killed)	9·1	35 100

An indication of the reliability of the ^{131}I-label as a marker for extracellular antibody was obtained from an experiment in which a rabbit was used which had been simultaneously immunized by intravenous injection both of pneumococci type 3 and alum-precipitated ovalbumin. The rabbit received an intravenous injection of a ^{131}I-labelled mixture of anti-capsular polysaccharide and anti-ovalbumin 2 days before extraction of the antibodies from lung and bone marrow in the usual way. Table 4 shows that although the absolute levels of the two antibodies differed widely, the percentage of extracellular antibody in the lung and bone marrow agreed closely for both kinds of antibody.

Illustrative experiment

In order to show how the results were arrived at, a typical experiment is summarized below. A rabbit, hyperimmunized against pneumococci type 3, was injected intravenously with about 25 mg. of a concentrate of rabbit γ-globulins labelled with ^{131}I, in which 85 % of the protein was specific antibody to capsular polysaccharide. The total activity injected was 91×10^6 counts/min. After 3 min., a blood sample was taken, and 1 ml. of plasma was found to contain $0{\cdot}892 \times 10^6$ counts/min. The plasma volume was thus 102 ml. The plasma equivalent of lymph and plasma is about 2·5 times this value (Cohen *et al.* 1956). Further small blood samples were taken daily, and the amounts of antibody and its radioactivity were measured after specific precipitation. The results, given in Table 5, show: (*a*) that although the antibody level fell slightly, the animal was producing antibody almost as fast as it was being destroyed, i.e. it was in an approximately steady state; and (*b*) that after 48 hr., assuming that equilibration between plasma and lymph had occurred by then, the rate of disappearance of the labelled protein was 8 %/day, consistent with the turnover rate of about 10 %/day observed by Humphrey & McFarlane (1954). These figures permit an approximate calculation of the daily rate of antibody production at the time of the experiment, namely:

wt. of antibody produced daily (mg.)
= fraction renewed × concn. of antibody × plasma equiv. of plasma and lymph
$= 0{\cdot}1 \times 9{\cdot}1 \times 250 = 228.$

Three days after the administration of ^{131}I-labelled proteins, 21 μc (0·5 mg.) of algal [^{14}C]-protein hydrolysate was injected intravenously and 2 hr. later the animal was perfused, and its antibody obtained from its tissues as described above.

In Table 6 are given the amounts and radioactivities of the antibody precipitates obtained from various tissues and from the serum. These figures include a correction for the extracellular antibody, but the correction factors given in Table 1 have not been applied.

Recovery of antibody from tissue extracts

In Table 7 are recorded the amounts of antibody recovered in typical experiments, together with the values adjusted to allow for extracellular antibody. It will be seen that with few exceptions the lungs contained considerably more antibody than the other tissues, even when allowance is made for the fact that only part of the bone marrow or lymph glands was examined. The tissue with the next highest content was the bone marrow. Spleen, lymph glands and appendix all contained intracellular antibody, but significant amounts were never recovered from liver or kidney.

Table 6. *Amounts and specific activities of antibody recovered in illustrative experiment*

The earlier procedure in this experiment is described in Table 5. Finally, 2 hr. before the same rabbit was killed, algal [^{14}C]protein hydrolysate (21 μc; 0·5 mg.) was injected intravenously. For subsequent procedures and for the corrections applied to obtain the values in the fourth and sixth columns, see the text and Table 3 respectively.

Tissue	Antibody recovered	Specific ^{131}I activity (counts/min./mg. of antibody)	Extracellular antibody (%)	Measured specific ^{14}C-activity (counts/min./mg. of C)	Corrected specific ^{14}C-activity of intracellular antibody (counts/min./mg. of C)
Lung	8·9 mg.	16 800	45	768	1220
Bone marrow	3·5 mg.	15 300	44	259	375
Spleen	1·5 mg.	5 320	15	1160	1340
Lymph glands	0·5 mg.	19 000	54	136	174
Appendix	0·5 mg.	11 900	34	118	126
Serum at 3 min.	9·1 mg./ml.	35 100	—	2	—
Serum at 2 hr.	9·1 mg./ml.	—	—	105	—

Table 7. *Amounts of antibody extracted from the tissues of hyperimmune rabbits after perfusion*

In each case column *A* gives the actual amounts of antibody precipitate recovered, and column *B* the amount which was calculated to be truly intracellular, as described in the text and Table 3. No corrections are made for losses in recovery (see text and Table 1). Since only about 6 g. of bone marrow and 2 g. of lymph glands were examined, the figures for these tissues should be increased four to five and two to three times respectively.

Rabbit no.	Serum antibody (mg./ml.)	Total antibody (mg.) Lung		Bone marrow		Spleen		Lymph glands		Appendix	
		A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
2	9·1	8·9	4·9	3·5	2·0	1·5	1·3	0·5	0·3	0·5	0·3
5	7·5	3·6	1·3	2·0	0·7	0·2	0·1	0·6	0·1	0·2	0·1
21	5·2	6·2	4·2	3·2	0·6	0·4	0·2	0·2	0·1	0·2	0·2
23	6·0	1·2	0·5	2·6	1·6	1·5	1·3	0·3	0·1	0	0
24	10·0	9·6	6·5	1·6	0·2	0·3	0·03	1·0	0·4	1·0	0·8
25	9·7	35·2	28·2	3·2	0·6	0·4	0·2	0·2	0·1	0·2	0·2
44	10·8	32·6	28·4	Not done		1·4	1·1	Not done		Not done	
82	14·9	16·5	12·8	3·9	2·4	1·3	0·5	1·4	0·7	1·1	0·5

Table 8. *Specific activities of intracellular antibody extracted at various time intervals after injection*

The total radioactivity of the injected [^{14}C]amino acids was in all cases 21 μc. Activities are expressed as counts/min./mg. of C and are corrected for extracellular antibody.

Rabbit no.	Time	Plasma	Lung	Bone marrow	Spleen	Lymph glands	Appendix
46	15 min.	8	480	Not done	Not done	Not done	Not done
29	15 min.	2	1750	1890	5300*	1930*	1980*
44	32 min.	49	1840	Not done	3250	Not done	Not done
49	1 hr.	35	870	Not done	Not done	Not done	Not done
28	1 hr.	44	2320	2950	1420†	2460	3200*
24	1 hr.	36	2160	4000*	8700*	2100	1550
19	2 hr.	176	975	910	2650	178	360
2	2 hr.	103	1320	375	1340	174	126
47	2·2 hr.	278	1615	Not done	Not done	Not done	Not done
5	5 hr.	198	920	435	485*	Nil	447*
23	5 hr.	215	587	538	872	112*	No antibody
48	5 hr.	188	1260	Not done	Not done	Not done	Not done
21	23 hr.	143	286	280	156	347*	127*
25	23 hr.	267	438	66	158	192*	214*

* Amounts of intracellular antibody were very small, and the estimate could be out by a factor of 2.
† Estimate too low. Antibody precipitate was obviously contaminated with other material, and weight was about three times that expected. The value was probably nearer 5000.

There was surprisingly little correlation between the antibody content of the plasma and that of the tissues, but it must be remembered that the contribution of any organ to the circulating antibody depends not only on the amount present but on its turnover rate. This will be discussed below.

Specific activity of intracellular antibody

Table 8 lists the specific ^{14}C-activities of antibody in the tissue extracts, after correction for extracellular antibody, but not for contamination with inert material. Once again, there is considerable variation from rabbit to rabbit, which is due at least in part to variation in their immunological responses. Although the number of animals was small, the main trend in lung, spleen and bone marrow is clear enough. Whereas antibody in plasma became increasingly labelled after 15 min., reaching a peak somewhere between 2 and 5 hr., antibody labelling in the tissues was already very high at 15 min., reached a peak after about 1 hr. and remained higher than that in the plasma for at least 5 hr.

These results must be considered in conjunction with those relating to the specific activities of the plasma and tissue free amino acids. They show that antibody is being made in the tissues examined. They also show that the time taken for incorporation of plasma amino acids into antibody molecules within the cells is certainly very much less than 15 min., even though the amounts of labelled antibody which reach the plasma within this period are insufficient to affect the radioactivity of protein in the plasma pool.

RESULTS OF AMINO ACID STUDIES

Specific activities of free amino acids in plasma after administration of algal-protein hydrolysate

Comparison of the methods. The turnover of any particular amino acid in the plasma is governed by several factors—notably by the number of metabolic pathways available for its synthesis or degradation and the amounts entering from exogenous and endogenous sources. Furthermore, the contribution of any amino acid to the total free amino acids of plasma or of intracellular fluid, on the one hand, and the proportion incorporated into antibody, on the other, will vary for each amino acid. It would therefore have been ideal, but immensely time-consuming, to have studied several different ^{14}C-labelled amino acids individually, by specific isolation and measurement of their radioactivities. We attempted to avoid the need for this by using the mixture of [^{14}C]amino acids found in *Chlorella*-protein hydrolysate, a mixture which contains all the amino acids found in rabbit-serum proteins (except for tryptophan destroyed during hydrolysis) in proportions not unlike those in whole-liver protein. It was reasoned that although some of the injected radioactive amino acids would be diluted more extensively or metabolized more rapidly than others, the average radioactivity of the free amino acids in the plasma, in the intracellular pool, and in the antibody synthesized from it, should be similar.

As already described under Methods, two procedures were used for estimating the specific activity of free amino acids in plasma. In the first the non-protein free amino N was estimated colorimetrically and this value was multiplied by 4·4 to approximate to the corresponding weight of amino acid C. [The figure calculated for *Chlorella*-protein hydrolysate from the data of Catch (1954) is 4·3, and the corresponding figure from published data on the free amino acids of rabbit plasma is 4·4.] Carbon specific activities were measured, after dilution with inert glycine, and the mean amino acid C specific activities were calculated on the assumption that the radioactivity came solely from amino acids. This method was convenient, but was based on an assumption which ceased to be strictly true once other radioactive metabolites had been formed.

The second method, namely, specific liberation of terminal carboxyl C with ninhydrin, was less convenient but its results were independent of the presence of radioactive non-amino acid metabolites, and are therefore more reliable. The two methods were directly compared on plasma samples taken from three rabbits 0·25–5 hr. after injection of amino acids, and the free amino acid C specific activities by the second method were 57–71% (mean 63%) of those by the first method. In Table 9 are given the results when [^{14}C]amino acids were administered to two series of rabbits and the plasma-free amino acid specific ^{14}C-activities were measured in one series by the glycine-dilution method and in the other by the ninhydrin-CO_2 method. During the period 0·25–5 hr. the results were similar to those obtained by direct comparison on the same plasmas, namely, that on the average the true specific activities were 65% of those obtained by the glycine-dilution method. This relationship was not exact, nor was it necessarily true outside this period of time (in fact, after 1 min., before a significant quantity of metabolic products had appeared in the plasma, the two methods agreed). It could be used as an approximation, however, in later calculations.

Time course of free amino acid specific activity in plasma. In Table 10 are summarized the values for the free amino acid C specific activities, obtained by the dilution method, at various time intervals after injection of [^{14}C]amino acids.

Table 9. *Comparison of specific activities of carbon in free amino acids of plasma, measured by the ninhydrin-carbon dioxide method and by the glycine-dilution method*

Rabbits were injected with varied amounts of an algal [^{14}C]protein hydrolysate. Specific activities given below have been adjusted to correspond with a dose of 21 μc/rabbit, and are therefore comparable with those in Table 8. Numbers of rabbits used to obtain a mean value are given in parentheses. Separate series of animals were used for each method. For reasons explained in the text the ninhydrin-CO_2 method is the more specific. Comparison of the results from the two methods gave the percentages shown in the final column; the average value in the period shown is 66 %. Procedures for separation of free amino acids from protein were different in the two methods.

Time of bleeding after injection (min.)	Ninhydrin-CO_2 method mean specific activity (counts/min./mg. of C)	Glycine-dilution method: Mean specific activity (counts/min./mg. of C)	Glycine-dilution method: Radioactivity from α-amino acid C only (%)
15	10 800 (2)	16 000 (2)	68
30	6 300 (2)	10 400 (2)	61
60	5 350 (2)	6 760 (4)	79
120	2 500 (2)	3 500 (3)	72
300	845 (1)	1 700 (2)	50

Table 10. *Plasma-free amino acid carbon specific activities (counts/min./mg. of carbon) at various times after injection of 21 μc of mixed [^{14}C]amino acids*

Values were obtained by the dilution method. Those from 15 to 360 min. should be multiplied by 0·65 to give the true values (see text and Table 9). The 3 min. samples were in fact taken after approx. 3 min., which partly accounts for the variable values. The specific activity at zero time was $2{\cdot}54 \times 10^6$; this value was obtained by adding 21 μc of mixed [^{14}C]amino acids to 100 ml. of rabbit serum.

Rabbit no.	Time (min.): ¼	1¼	3	15	30	60	120	240	300	360
30*a*	465 000	—	—	—	—	—	—	—	—	—
29*a*	—	193 000	—	—	—	—	—	—	—	—
27*a*	—	189 000	—	—	—	—	—	—	—	—
46	—	—	28 800	15 000	—	—	—	—	—	—
44	—	—	27 300	17 000	9 450	—	—	—	—	—
49	—	—	22 100	—	—	3 620	—	—	—	—
24	—	—	36 200	—	—	10 900	—	—	—	—
28	—	—	32 000	—	—	7 100	—	—	—	—
47	—	—	44 500	—	—	6 150	4000	—	—	—
19	—	—	34 300	—	—	—	4130	—	—	—
2	—	—	27 800	—	—	—	2680	—	—	—
48	—	—	29 600	—	—	—	—	—	2940	—
5	—	—	106 000	—	—	—	—	—	1500	—
23	—	—	59 300	—	—	—	—	—	1950	—
21	—	—	34 600	—	11 400	—	3200	—	—	950
25	—	—	29 700	—	—	6 030	—	2040	—	—

Except for those at less than 3 min. (which are included for completeness) the values were all obtained in those rabbits which were used for antibody studies. As already mentioned, the true values at 15–300 min. are probably 0·65 times those given in the table; in Fig. 1, which illustrates graphically the time course of the specific activity of free amino acid C this correction has been made.

There is considerable variation between individual rabbits, part of which is due to inexact measurement of the shorter time intervals, since we were unaware how rapidly the specific activity was changing. The general picture for the mixed amino acids, however, is very similar to that observed by Henriques, Henriques & Neuberger (1955) for glycine and by Barry (1952) for lysine and tyrosine. What is apparently a new observation is the extremely rapid fall during the first 3 min. Even when blood was obtained by opening the heart 45 sec. after injection (the shortest period examined) the free amino acids in the plasma contained only 17 % of the injected radioactivity, yet simultaneous injection of the dye Pontamine Sky Blue 6BX (which binds to plasma albumin) showed that complete mixing of the injected material with the plasma had taken place.

Specific activity of free amino acids in the tissues. As already described, the specific activities of the free amino acids in various tissues, at different time intervals after giving [^{14}C]amino acids, were measured in a separate series of rabbits. In Table 11 the results are set out.

After correction to a 'standard' dose of 21 μc, the plasma-free amino acid C specific activities lie reasonably close to the curve shown in Fig. 1. The tissue-free amino acid C specific activities, expressed as a percentage of those in the plasma, increased rapidly during the first 15 min. and thereafter increased slowly or remained very approximately steady for the next 4 hr. When the absolute specific activities for tissue amino acids are plotted (Fig. 2), although erratic, they show curves like that of the plasma-free amino acids but very much flattened. Since the cellular composition of the different tissues is complex and, except for liver and appendix, varies with the degree of immunization, animal variation is probably a sufficient explanation of the erratic distribution of the points. The low specific activities of the amino acids in spleen and bone marrow, compared with liver, were surprising for such metabolically active tissues. They may indicate that a considerable proportion of the intracellular free amino acids were derived from unlabelled sources (e.g. plasma proteins), or that cells were present (e.g. erythrocytes) whose internal free amino acids equilibrated only slowly with those in plasma. In the bone marrow there may also have been a significant breakdown of cell protein, during the minutes required to remove the tissue into liquid air. The amounts of free amino acid carboxyl C recovered from 1 g. of the moist perfused tissues corresponded approximately to the following amounts of free amino acid (calculated on the assumption that terminal carboxyl carbon composed 10 % of the amino acid mixture): liver, 2 mg.; appendix, 2·8 mg.; spleen, 2·4 mg.; bone marrow, 2·2 mg.; lung, 1·8 mg. There was, however, a considerable scatter in the estimate for the last three.

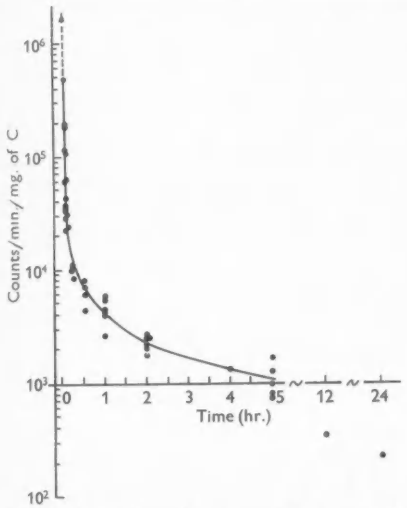

Fig. 1. Specific radioactivity (counts/min./mg. of C) of plasma-free amino acids in rabbits at various times after intravenous administration of 21 μc of *Chlorella* [^{14}C]-protein hydrolysate. The values are taken from all experiments, but those obtained by the dilution method have been corrected to agree with the ninhydrin-CO_2 method (see Methods and Table 9). The value at zero time was obtained by adding 21 μc of the labelled amino acids to 100 ml. of rabbit serum.

It is possible to estimate graphically from Fig. 2 the approximate mean specific activities of free amino acid C of the tissues over any given period of time. The values so obtained, however, inevitably apply only to the totality of cells present in the tissue and not to any single type.

DISCUSSION

The main facts demonstrated above are that, from tissues of hyperimmunized rabbits, intracellular antibody can be extracted and measured; that,

Table 11. *Specific activities of free amino acid carbon in rabbit tissues at various times after administering the* [^{14}C]*amino acid mixture*

Specific activities were measured by the ninhydrin-CO_2 method. Two rabbits were used at each time.

Expt.	Radioactivity injected (μc)	Time after injection (min.)	Specific activity of plasma-free amino acid (counts/min./mg. of C)	Relative specific activity of tissue-free amino acid (% of plasma values)				
				Lung	Bone marrow	Spleen	Appendix	Liver
39–40	1·5	5	3820	20	7	15	12	29
41–42	2·4	10	2280	53	15	19	27	43
84–85	2·5	15	1050	52	14	32	39	51
3–5	6	30	1540	31	45	34	81	81
2–6*a*	8	60	1710	55	66	20	43	17*
1–6	14	120	1360	118	66	86	42	74
4–24	25	270	1050	77	—	29	108	65

* Recovery of carboxyl carbon was much greater than expected, and this value is doubtful.

after a single intravenous injection of ^{14}C-labelled amino acids, such antibody becomes labelled rapidly, being already highly labelled after 15 min., and remaining so for 1–2 hr., after which the radioactivity slowly declines; and that significant labelling of antibody in plasma is not detectable for 20–25 min., after which it increases sharply, reaching a peak between 2 and 4 hr.

The interval before labelled proteins appear in the blood stream has been termed the 'transit time' by Green & Anker (1955) and is regarded by them as probably implying that there is some specific delay between the uptake of amino acids by cells, their appearance and synthesis into protein and the release of the protein from the cells into the blood. Since intracellular antibody was found to be extensively labelled in much less than the 'transit time', it is unlikely that the main delay arises at the stage of incorporation of amino acids into soluble protein. Although there may be a lag between formation of new protein molecules and their appearance in soluble form in the cells, this lag must be relatively short. In our view the time course of labelling of plasma antibody can be approximately accounted for by a continuous release of antibody (initially of low radioactivity, because of dilution with a pre-existing unlabelled intracellular pool) from cells in various tissues into the surrounding fluid, followed by passage into the blood stream, to some extent directly across capillary walls, but more commonly by lymphatic pathways of varying length; the whole process being superimposed upon a steady metabolic elimination from the blood stream.

We were interested to see whether our data could be used to calculate the actual turnover of antibody in different tissues, and to assess their relative contributions to antibody synthesis *in vivo*. Before doing so, however, it is necessary to consider the gross overall mechanisms whereby amino acids could be incorporated both into intracellular and extracellular antibody. Without prejudice to the

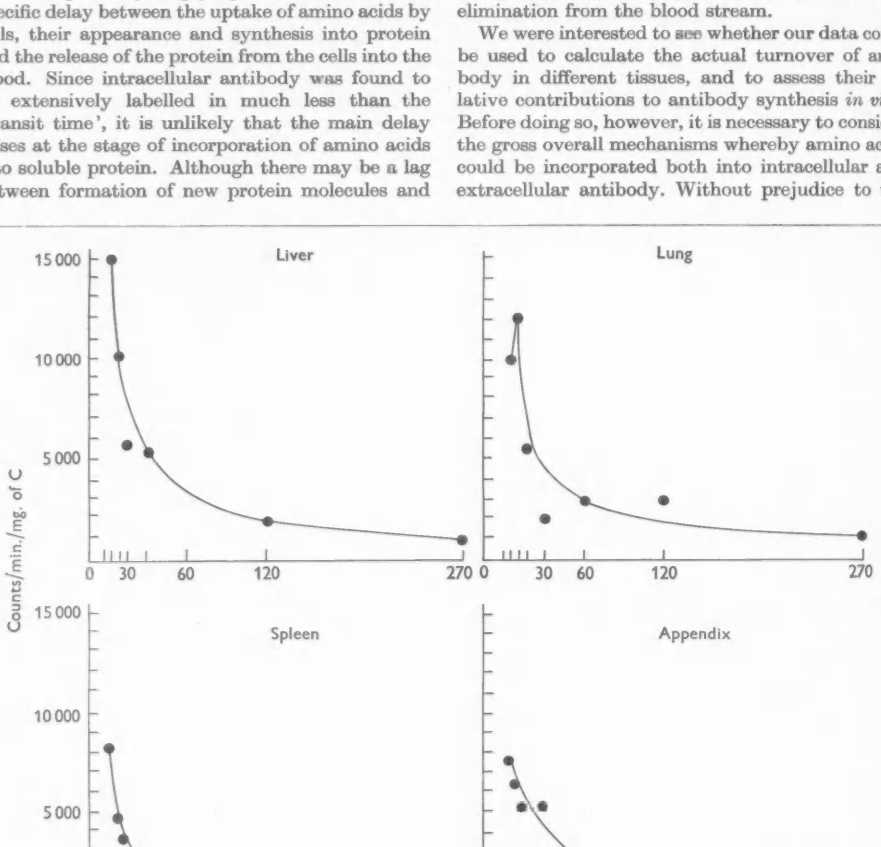

Fig. 2. Specific radioactivity of intracellular free amino acids in different tissues at various times after intravenous administration of 21 μc of *Chlorella* [^{14}C]protein hydrolysate. Each point is a mean value from two or more animals. For further details, see text and Table 11.

details of how amino acids are converted into protein, or to the mechanics of the process of secretion, there are two main possibilities. One is that synthesis of protein occurs close to the cell surface, and that newly formed molecules diffuse both outward into the tissue fluid and inward into the cell, i.e. the secreted antibody does not come from the intracellular antibody pool, and its specific radioactivity therefore resembles that of the intra- or extra-cellular free amino acids rather than that of intracellular antibody. The second possibility is that antibody is formed continuously within the cells from precursors in the free amino acid pool, and that the newly formed molecules mix with a pool of intracellular antibody from which a steady loss occurs into the surrounding fluid. Experiments on antibody production in isolated perfused lungs (B. A. Askonas & J. H. Humphrey, unpublished work) suggest that the second mechanism is the more probable. It also provides a better explanation of the time course of appearance of radioactive antibody in the blood stream, besides lending itself more readily to simple mathematical treatment. We have therefore adopted the second hypothesis in the calculations given below.

Calculation of turnover rates of intracellular antibody

Before any calculations are attempted from our data certain assumptions must be made. One is that estimates of the specific activities of intracellular free amino acid made in complex tissues are valid for the particular cells which make antibody. There is at present no way of checking this. A second is that the ^{14}C-content of newly formed antibody is the same as the ^{14}C-content of the amino acid mixture in the intracellular free amino acid pool as estimated by the ninhydrin-carbon dioxide method. Although all the amino acids administered were equally labelled, and the mixture contained all the amino acids (except tryptophan) in proportions roughly resembling those in a whole-liver hydrolysate (Mütung & Wortmann, 1954), this assumption is unlikely to be strictly true. The reason for this is that free amino acids in tissues contain a very high proportion of glutamic acid and glutamine. If taurine, which would not be estimated by our methods, is excluded, glutamic acid and glutamine account for a little over half the free amino acids in liver, pancreas or kidney of the cat (Tallan, Moore & Stein, 1954), whereas glutamic acid accounts for only about 7 % of the amino acids of the *Chlorella* protein used (Catch, 1954) and about 11 % of the amino acids of rabbit antibody (Smith, McFadden, Stockell & Buettner-Janusch, 1955). The specific activity of the free amino acids, other than glutamic acid, in the intracellular pool may therefore be considerably underestimated. A third assumption is that all the antibody-forming cells were continuously active during the experiments, i.e. that intracellular antibody was homogeneous and did not consist to any great extent of metabolically inert pre-formed antibody which was stored in some cells and not secreted. This assumption is also probably not strictly true; certainly histological sections examined for antibody by the fluorescent-antibody technique (Coons *et al.* 1955) showed some cells which contained antibody diffusely throughout the cytoplasm and others which showed antibody in compact granular masses. Furthermore, experiments on the isolated lungs of hyperimmunized rabbits perfused with [^{14}C]amino acids (B. A. Askonas & J. H. Humphrey, unpublished work) showed that in about half the cases intracellular and secreted antibody had closely similar radioactivities, which is consistent with the third assumption, but in other cases there was evidence that metabolically inert antibody was also present. Lastly, it is assumed that antibody production is in a steady state, i.e. that the rate of formation of new antibody is equal to the rate of secretion into the tissue fluids.

Suppose that antibody-forming cells in a tissue contain Q mg. of antibody, and that the rate of secretion is Q/K mg./hr. (which is, by assumption, continuous and equal to the rate of synthesis); K will then be the turnover time of the intracellular antibody.

At t hr. after the administration of labelled amino acids let the specific activity of intracellular free amino acid and antibody be respectively x and y counts/min./mg. of C. Suppose that all the antibody secreted in t hr. (Qt/K mg.) were combined, then its specific activity would be

$$\left(\int_0^t y\,\mathrm{d}t\right)\Big/t,$$

so that total radioactivity in antibody after t hr.

$$= Qy + \left(Q\int_0^t y\,\mathrm{d}t\right)\Big/K. \qquad (1)$$

Similarly,

total radioactivity added

$$= \left(Q\int_0^t x\,\mathrm{d}t\right)\Big/K. \qquad (2)$$

These two quantities must always be equal; hence, by differentiation,

$$Q\,\mathrm{d}y/\mathrm{d}t + Qy/K = Qx/K,$$

and

$$\mathrm{d}y/\mathrm{d}t = (x-y)/K. \qquad (3)$$

This equation could be used to find K, provided

that the parameters were known. In the whole animal the amino acid specific activity is changing with time (Fig. 2 and Table 10), and no second theoretical equation relating dy/dt to x or y is known. Only a graphical solution is therefore possible. This, however, requires a knowledge of the change in specific radioactivity of intracellular antibody with time, which can be measured only for a series of animals, since each estimation involves killing one of them. The values for lung antibody from different experiments have therefore been combined and a curve has been constructed which could be applied to an 'ideal' hyperimmune rabbit (Fig. 3, in which the values have been corrected for 'purity'). It is recognized that deductions made from such a curve are not applicable to any single experiment and that they must be very approximate. Nevertheless, they are of some interest since they may indicate at least the order of magnitude of the turnover time.

Combining the values from Figs. 2 and 3, and substituting in equation (3), we obtain estimates for K relating to antibody turnover in lung which are given in Table 12. If the theory and the experimental results agreed, the curve of intracellular antibody specific activity should show a maximum at a time equal to K, at which x and y should be equal. Although the curves could obviously have been drawn somewhat differently, it seems that a value for K of about 2 hr. is of the right order. It can, however, be regarded only as very approximate, and will be an underestimate if the specific activity of the amino acids in the intracellular pool has been underestimated.

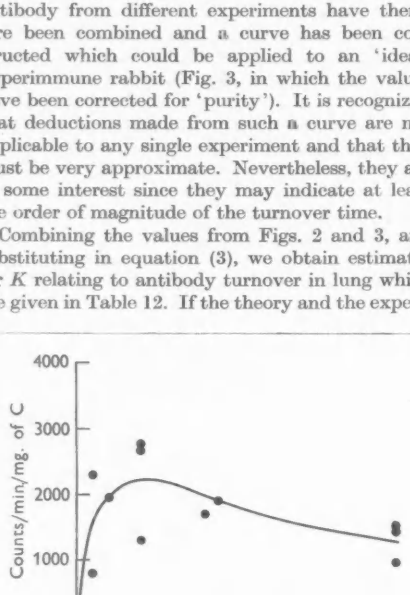

Fig. 3. Specific radioactivity of intracellular antibody in the lungs at different times after intravenous administration of 21 μc of *Chlorella* [^{14}C]protein hydrolysate. The values have been corrected for extracellular antibody and for 'purity' (see text).

Similar calculations are not possible for the other tissues, because the experimental values are too few and, owing to the very small weights of antibody recovered, several are not sufficiently reliable for deduction of quantitative conclusions. The turnover time of antibody in spleen is probably rather shorter than that of antibody in lung, since the radioactivity of the former was greater during the first hour after administration of the label, but after 5 hr. or more was less. The turnover times in bone marrow, lymph glands and appendix are probably similar to that in lung.

It is possible to test whether these estimates of turnover time are reasonable by taking them in conjunction with the measurements of intracellular antibody (Table 7) corrected for partial recovery (Table 1), and comparing the calculated rate of antibody secretion with that required to maintain the observed levels of antibody in serum. A 2·5 kg. rabbit, in a steady state, with a serum-antibody level of 10 mg./ml., has about 2·4 g. of antibody in the blood and tissue fluid, and replaces in 24 hr. about 10% of its circulating antibody, i.e. about 240 mg. If the average turnover time of intracellular antibody in all tissues were 2 hr. this would require 20 mg. of total intracellular antibody, i.e. the total amount of intracellular antibody should be about equal to the quantity of antibody contained in 2 ml. of serum.

The actual recoveries of intracellular antibody from lung, bone marrow, spleen, lymph glands and appendix, after allowing for partial recovery and assuming a total of 30 g. of active bone marrow and 6 g. of lymph glands, lie between 72 and 200% (mean 144%) of the antibody in 2 ml. of serum.

Table 12. *Calculation of turnover time of intracellular antibody in lung*

The turnover time, K, is calculated from the equation $dy/dx = (x-y)/K$ (see text). Values of x and y were obtained from the curves shown in Figs. 2 and 3 respectively. Approximate values for dy/dt were obtained graphically from the curve shown in Fig. 3.

Time (hr.)	x (counts/min./mg. of C)	y (counts/min./mg. of C)	dy/dt	K (hr.)
$\frac{1}{4}$	6000	1500	2500	1·8
$\frac{1}{2}$	4500	2000	1150	2·2
$\frac{3}{4}$	3500	2100	600	2·3
2	1800	1950	−350	0·4
3	1400	1700	−200	1·5
5	900	1300	−170	2·4

This finding suggests that the estimates are of the right order, although an average turnover time of 3 hr. would give better agreement.

Sites of antibody production

This series of experiments was confined to rabbits hyperimmunized by intravenous injection of a particular bacterial antigen, and the results are not necessarily true for other species, or for immunization by other antigens or by other routes. With this proviso, it may be said that the chief source of antibody in most animals was the lung tissue, and that the next most important source (occasionally the main source) was the bone marrow. The spleen contributed less than the bone marrow, but usually more than the lymph glands and appendix. The liver and kidneys never contained significant quantities of antibody. There was, however, a marked variation from animal to animal in the contribution by the other tissues examined. Apart from their emphasis on the lungs, which have not usually been regarded as important sources of antibody, the conclusions agree generally with those of other workers. They are also in good agreement with results obtained in rabbits by incubation of tissue slices with [^{14}C]amino acids *in vitro* (B. A. Askonas & J. H. Humphrey, unpublished work). Other results obtained by the *in vitro* method (Askonas & White, 1956) on guinea pigs which received ovalbumin with adjuvant in the foot pad, showed a quite different distribution of antibody synthesis, nearly all being made in the bone marrow and lymph glands, with very little in the spleen and none in the lung.

Turnover of plasma-free amino acids

An observation of some interest, although only incidental to this work, is the extremely rapid turnover of plasma-free amino acids. Three-quarters of a minute after injection of labelled amino acids there remained in the plasma less than 20%; at 3 min. only about 2%; and at 5 min. only 1%. A large part of the drop in radioactivity of the plasma-free amino acids is due to very rapid diffusion from the blood into the extravascular fluid. If such diffusion occurred freely and completely into the whole of the extravascular water (the volume of which may be taken as approximately 25% of the rabbit's weight or about six times the plasma volume; Manery, 1954), the radioactivity would be expected to drop to 16% in the absence of any removal into cells. Thus neglecting changes which took place during separation of plasma *in vitro*, and any return from cells to extracellular fluid, the observed drop to 1% in 5 min. must be due to the entry of extracellular amino acids into cells, at a rate corresponding to a half-life of not more than 1·25 min. If the amino acids throughout the extracellular space were all equally available for exchange with cells, this would imply that in a 2·5 kg. rabbit, whose plasma and extracellular fluid contained amino acids at a concentration of, say, 40 mg./100 ml., some 100 mg. of amino acids entered (and left) the cells every minute. The extracellular water is, in fact, far from homogeneous, and exchange of amino acids between it and the cells is relatively slow in muscle (Henriques *et al.* 1955) and is likely to be slow in the relatively acellular connective tissue and skin. The extracellular water of these tissues comprises between them some 60–65% of the whole. If diffusion of labelled amino acids from these spaces back into the bloodstream were slow compared with the rate of disappearance of the label into cells in other tissues, the result would be that the above estimate of the rate of exchange of amino acids between cells and their surroundings was too high.

It can be seen from Fig. 1 that after about 5 min. the rate of decline of free amino acid specific activity in the plasma diminishes markedly, presumably because re-entry of the label from the cells becomes significant, and by 12 hr. it has become quite slow. The immediate fate of the labelled amino acid molecules when they leave the blood stream is of considerable interest. In a recent review McFarlane (1957) points out that inside 5 min. more than 90% of injected amino acid radioactivity leaves the lymph and plasma, conclusions based on calculation rather than direct measurement, but closely similar to our own. It is a commonly accepted hypothesis that, after mixing with the plasma and tissue fluids, the injected molecules come rapidly into equilibrium with an intracellular free amino acid pool, which is in turn in equilibrium with the tissue proteins. McFarlane (1957) regards the existence of a true intracellular free amino acid pool of any size as improbable, and prefers the hypothesis that the pool consists in fact of a labile intracellular protein which is in process of rapid exchange with free amino acids. Provided that equilibrium between labile protein and intracellular free amino acids were sufficiently rapid, and that the protein were so unstable as to become largely hydrolysed to free amino acids during experimental handling of the tissues, either hypothesis could accommodate our results. As will be shown, however, the size of the intracellular pool, whether of free amino acids or of labile protein, must be quite considerable and in the absence of direct evidence for any labile protein in large enough amounts, it is simpler to consider a real pool of intracellular free amino acids. Henriques *et al.* (1955), using a technique which might be expected to avoid post-mortem changes, found that in rabbits injected with

[^{14}C]glycine maximum labelling of tissue proteins does not occur until after an hour or more. Even in mice, Borsook, Deasy, Haagen-Smit, Keighley & Lowry (1950) found 30–80% of visceral-intracellular radioactivity to be in non-protein form 10 min. after injection of various labelled amino acids. Maximum labelling of plasma proteins in the rabbit takes about 3 hr., and it is therefore clear that loss of ^{14}C by incorporation into any relatively stable proteins cannot be of much importance in considering what happens to the labelled amino acids during the first few minutes after intravenous injection of tracer doses. As has already been stated, within 5 min. the specific radioactivity of free amino acids in the plasma has fallen to about one-hundredth of the calculated initial value. Since only a drop of about sixfold can be accounted for by mixing with amino acids in the extravascular extracellular fluid, the effective size of the rapidly equilibrating intracellular free amino acid pool must be at least 16 times as great as the total extracellular free amino acid pool (or 100 times the free amino acid pool in the plasma). If there is twice as much intracellular water as extracellular (approximate value from Manery, 1954), this would imply that on average cell water contains a concentration of readily exchangeable free amino acids eight times that in the plasma. This conclusion is in approximate agreement with the finding of Christensen, Streicher & Elbinger (1948) that in guinea pigs the concentration of α-amino nitrogen in liver water is about 16 times and in muscle about six times that in the plasma. A further deduction is that 5 min. after injection of 21 μc of algal-protein hydrolysate the specific activity of the intracellular amino acids would average not more than about 25 000 counts/min./mg. of carbon. The effects of dilution due to a rapid intracellular turnover of unlabelled glutamic acid; of disappearance of the labelled amino acids into intracellular protein; or of mixing with unlabelled amino acids produced by autolysis *post mortem* will all be to lower this figure. Thus the fact that the observed values corresponded to 15 000 counts/min./mg. of carbon in liver, 10 700 in lung and 8000 in spleen may also be taken to indicate that our reasoning, despite the numerous assumptions and approximations, is in essence valid.

SUMMARY

1. Intracellular antibody was recovered from tissues of rabbits hyperimmunized by intravenous injections of type 3 pneumococci, and perfused free of blood at various time intervals after administration of ^{14}C-labelled amino acids. Allowance was made for pre-formed extracellular antibody by injecting ^{131}I-labelled antibody 2–3 days beforehand, and allowing it to come into equilibrium with antibody in the extracellular fluid.

2. ^{14}C-Labelled antibody was found in lungs, bone marrow, spleen, lymph glands and appendix (but not in liver or kidneys) within a few minutes of adminstering the label, and before ^{14}C was detectable in plasma antibody.

3. The amounts of intracellular antibody recovered from tissues were: lung > bone marrow ≥ spleen ≥ lymph glands ≥ appendix.

4. The time course was determined of the specific ^{14}C-activities of intracellular antibody and, of the intracellular free amino acids in various tissues, and of the plasma-free amino acids. From the results the 'turnover time' of intracellular antibody in the lung was calculated to be of the order of 2 hr.

5. From consideration of the total amounts of intracellular antibody recovered, and of the daily production of antibody required to maintain the observed plasma levels, the average 'turnover time' of all tissue antibody was calculated to be of the order of 3 hr.

6. Consideration of the time course of plasma-free amino acid specific activities during the first few minutes after injection of a mixture of ^{14}C-labelled amino acids suggests (for a 2·5 kg. rabbit) that (i) the rate of exchange of amino acids between cells and tissue fluids is of the order of 100 mg./min., and (ii) the size of the 'intracellular free amino acid pool' with which this rapid exchange occurs is of the order of 100 times the plasma-free amino acid pool.

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The Excretion of Microgram Doses of Hexoestrol by Rabbits and Rats

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Extremely small doses of all the closely related synthetic female sex hormones are capable of producing a physiological response in laboratory animals. The effective subcutaneous dose of *meso*-hexoestrol for a rat is 0·2 μg. (Dodds *et al.* 1944), and from work on mice it appears that about a two-hundredth of the subcutaneous dose is effective when administered locally to the vagina (Emmens, 1940–41). The metabolism of such extremely potent substances is clearly of considerable interest, and this interest is heightened by their well-known use in cancer, particularly carcinoma of the prostate. Treatment with oestrogens causes a temporary regression of the malignancy, which, however, recurs after varying intervals of time when they are no longer effective. This was noted by Smith & Williams (1948), who suggested that the failure of continued treatment might be due to an alteration in the metabolism of the drug. Metabolism of female sex hormones is also of interest from the point of view of the induction of artificial lactation and of the fattening of cattle.

As pointed out by Hanahan, Daskalakis, Edwards & Dauben (1953) studies on the metabolism of oestrogens have been carried out in the main through the massive doses which were necessitated by the relatively insensitive methods available for their detection in the excreta and tissues of the experimental animals. The isotopic tracer method is particularly suited to the elucidation of this problem because of the very small amounts of radioactive substances which can be detected. Such a method was applied by Albert, Heard, Leblond & Saffran (1949), who studied the metabolism of [^{131}I]iodo-α-oestradiol after injection into mice at doses ranging from 1·7 μg to 2 mg. The iodo-compound, unlike the parent oestradiol, however, is not physiologically active even at doses as high as 100 μg. and this, coupled with the fact that the actual doses used were mostly very much higher than was the effective dose of oestradiol itself, somewhat detracts from the value of the work. However, these workers note that the outstanding feature of the metabolism of their compound was accumulation in the gastrointestinal tract, which agrees with what has been found for physiologically active oestrogens.

Twombly (1951) and Twombly & Schoenewaldt (1951) have studied the metabolism in female mice of ^{14}C-labelled stilboestrol administered in doses of about 2 mg., which is more than a thousand times the effective physiological dose. They found that most of the dose was excreted in 21 hr., 70–85% by way of the faeces and 15–30% by way of the

urine. There was no selective absorption in any organ other than the liver and complete oxidation was negligible. Budy (1955) studied the metabolism of ^{14}C-labelled oestrone administered to female mice in doses of 0·1 mg. She found it to be more slowly excreted than stilboestrol, with some evidence of localization in liver, adrenals, lymph nodes, blood and bone, but none in reproductive organs.

Hanahan *et al.* (1953) have approached more nearly to effective physiological dose levels in their work with ^{14}C-labelled stilboestrol. Their compound was labelled in the ethyl side chain and was administered to rats in 5 μg. doses. This is about ten times the median-effective dose but was necessitated by the low specific activity ($2{\cdot}5 \times 10^4$ counts/min./μg.) attainable by the use of ^{14}C-labelling. This specific activity was sufficient for gross-distribution studies, but would probably not be sufficient for a study of the more detailed metabolism that is ultimately required. Hanahan *et al.* found that a release of a conjugated derivative by way of the bile and excretion in the faeces is the major metabolic pathway, urinary excretion being negligible.

The present experiments have been carried out with tritium as a labelling agent. With tritium very much higher specific activities are attainable than with ^{14}C: the tritio-hexoestrol used in this work had a specific activity of more than 10^7 counts/min./μg. compared with only $2{\cdot}5 \times 10^4$ counts/min./μg. of ^{14}C-labelled stilboestrol as used by Hanahan *et al.* (1953). And whereas the specific activity of the preparation used by those workers probably approached the maximum attainable, a tritium-labelled hexoestrol higher in specific activity by another factor of 10 should be possible and its preparation is being planned. Tritium, of course, is inferior to ^{14}C in that it does not label a specific atom in the carbon skeleton of the compound being studied and in that the possibility of exchange or labilization reactions must be eliminated before positive conclusions can be safely drawn. But it has the overriding and important advantage of conferring very high specific activities which make possible the use of physiological doses and less.

EXPERIMENTAL

Tritium-labelled hexoestrol. This was prepared by hydrogenation of dienoestrol with tritium–hydrogen (nominally 10% of tritium) on a micro scale. A preliminary account of the method used has been published (Glascock, 1954*a*). From the method of preparation and purification it is believed to be generally labelled in stable positions. In the preliminary experiments, which were carried out before a technique for the chromatographic purification of the labelled material had been worked out, the hexoestrol was used after twice recrystallizing from benzene–light petroleum (b.p. 90–110°). It had m.p. 176–178°, i.e. 7° lower than that of the authentic material. It was stored in ethanol solution at a concentration of 270 μg./ml. In the main experiments the chromatographically purified material, m.p. 183–184°, was used. As this had a much higher specific activity than the earlier specimen it was stored in the dark at 4° in benzene solution at a low concentration (55 μg./ml.) to minimize chemical attack by any possible products of radiolysis of the solvent.

Assay of labelled hexoestrol. Both preparations were diluted with unlabelled carrier hexoestrol so that the dilution had a specific activity of the order of 2000 counts/min./mg. For the first preparation 0·095 ml. of the stock solution was diluted to 100 ml. with ethanol containing 94·1 mg. of unlabelled hexoestrol. This was added to avoid the manipulation of very dilute solutions, from which solute might be lost by adsorption or other means. In 1 ml. of this solution was dissolved 131·5 mg. of hexoestrol, the solvent was removed by evaporation under a jet of hydrogen and the residue dried to constant weight. The product had a specific activity of 1350 counts/min./mg. of combustion water, from which it was calculated that the hexoestrol in the stock ethanol solution had a specific activity of $5{\cdot}4 \times 10^5$ counts/min./μg. By a similar procedure the specific activity of the second preparation was found to be $1{\cdot}136 \times 10^7$ counts/min./μg.

Measurement of radioactivity. All the samples were burnt and the combustion water was converted into butane and counted in a gas counter as described by Glascock (1954*b*). Except for samples of very low specific activity, all samples of butane were divided into at least two portions, which were counted separately for at least 10 000 counts. The several values for specific activity so obtained usually agreed to within $\pm 1{\cdot}5\%$ of their mean. The whole of the butane from samples of very low specific activity (e.g. blood water, blood solids, skin) was put into the counter at once and counted for as long as possible, usually at least 1000 counts. The probable error of counting of these samples is therefore likely to be about $\pm 3\%$. From the specific activity of the butane that of the combustion water can be calculated. When this is known the specific activity of a pure compound such as hexoestrol can be calculated from its chemical formula, but that of a mixed substance such as faeces, urine or blood solids can be calculated only if its yield of water on combustion is known. This was ascertained where necessary by carrying out a combustion, condensing the water into a tube and weighing.

Administration to animals. In the preliminary experiments two virgin female rabbits R 1/54 and R 1/53 were used, of weights 2·24 kg. and 3·19 kg. R 1/54 received 1 μg. ($5{\cdot}4 \times 10^5$ counts/min.) and R 1/53 received 100 μg. ($5{\cdot}4 \times 10^7$ counts/min.) of labelled hexoestrol subcutaneously in 0·5 ml. of aqueous solution. The animals were kept in metabolism cages and allowed to feed freely on their normal diet. Urine and faeces were collected for the periods 0–48 hr. and 48–96 hr. The faeces were dried at 100°, ground and assayed for tritium. Since experiments had shown that freeze-drying of aqueous solutions of hexoestrol containing less than 0·1 μg./ml. resulted in substantial losses, presumably by sublimation, 1 mg. of carrier hexoestrol was added to the urine samples before freeze-drying. Samples of from 2 to 4 ml. were then freeze-

dried in 20 ml. tubes on a vacuum system to constant weight. The residue in the tube was scraped off the walls as completely as possible, well mixed and assayed for tritium.

Two rat experiments were set up, each with five virgin females. Each animal received 1 μg. ($1{\cdot}136 \times 10^7$ counts/min.) of chromatographically purified labelled hexoestrol subcutaneously in 0·2 ml. of arachis oil. They were kept in metabolism cages for the separate collection of urine and faeces and killed at 8, 24, 24, 32 and 48 hr. respectively after being injected. At death, samples of blood were withdrawn and freeze-dried, both residual blood solids and blood water, which is assumed to be representative of body water (see Tables 2 and 3), being assayed for tritium. Urine and faeces were treated and assayed for tritium as in the rabbit experiments.

RESULTS

Rabbit experiments. In Table 1 are shown the analyses and radioactivity measurements of urine and faeces from the two rabbits. Although the volume of urine passed by each rabbit during the two periods of collection is variable, the total of solids excreted was fairly constant at 12–15 g. (Table 1, column 5). These solids also showed little variation in composition, at least so far as is indicated by their water yield on combustion (column 6). The specific activity of this combustion water was low in the samples collected during the second 48 hr. period from the rabbit which had received only 1 μg., but in all determinations it was high enough for the counting rate of the butane to be reliably measured. For both rabbits the total amount of radioactivity excreted in the urine solids was much lower during the second 48 hr. than during the first, although this difference is greater for the rabbit which had received 100 μg. than for that which had received only 1 μg. Whereas the first rabbit secreted more than one-fifth as much during the second 48 hr. as it did during the first 48 hr., the second rabbit secreted only about one-thirtieth as much. This difference may be merely biological variation or it may be due to the difference in dose.

Both animals excreted a considerable amount of radioactivity in the faeces although the specific activity of that obtained during the second 48 hr. from the rabbit which had received 1 μg. was too low to measure. Whereas 32·6 % of the total dose was excreted in the faeces of the rabbit which had received 1 μg., only 22 % was excreted in the faeces of the one which had received 100 μg.

Table 1 also shows that 81·5 % of the 1 μg. dose and 63·26 % of the 100 μg. dose was recovered in the excreta, which leaves a substantial fraction in each experiment unaccounted for. Clearly much of this might still be in the animal, either in the form of hexoestrol or its major metabolites, or in the form of metabolic water. The urine water of the

Table 1. *Recovery of tritium-labelled hexoestrol from rabbits*

Rabbit R 1/54 received 1 μg. ($5{\cdot}4 \times 10^6$ counts/min.) and rabbit R 1/53 100 μg. ($5{\cdot}4 \times 10^7$ counts/min.) in aqueous solution subcutaneously.

Rabbit	Sample	Period of collection (hr.)	Quantity	Total solids (g.)	Combustion water from total solids: Yield (%)	Total (g.)	Specific activity (counts/min./mg.)	Total activity (counts/min./mg.)	Equivalent as hexoestrol (μg.)
R 1/54	Urine	0–48	290 ml.	12·8	39·3	5·02	43·4	$2{\cdot}18 \times 10^5$	0·403
		48–96	415 ml.	14·8	41·5	6·1	7·6	$4{\cdot}64 \times 10^4$	0·086
	Faeces	0–48	27·9 g.	—	52·0	14·5	12·15	$1{\cdot}76 \times 10^5$	0·326
		48–96	21·6 g.	—	52·0	11·0	0	0	0
								Total	0·815
R 1/53	Urine	0–48	360 ml.	14·0	39·0	5·46	3895	$2{\cdot}16 \times 10^7$	40·0
		48–96	605 ml.	12·8	41·0	5·25	125	$6{\cdot}55 \times 10^5$	1·22
	Faeces	0–48	50·8 g.	—	52·0	26·4	422	$1{\cdot}11 \times 10^7$	20·6
		48–96	28·3 g.	—	52·0	14·7	53	$7{\cdot}77 \times 10^5$	1·44
								Total	63·26

Table 2. *Recovery of tritium-labelled hexoestrol from rats*

Each rat received 1 μg. of hexoestrol ($1{\cdot}136 \times 10^7$ counts/min.) in arachis oil subcutaneously. Body water was taken as 80 % and whole blood as 6·7 % of the body wt.

					Combustion water from total solids				
Rat and body wt. (g.)	Time killed (hr.)	Sample	Quantity	Total solids in sample (mg.)	Yield (%)	Total (mg.)	Specific activity (counts/min./mg.)	Total activity (counts/min.)	Equivalent as hexoestrol (μg.)
P 1 (230)	8	Urine	16 ml.	321	47·3	151	6160	$0{\cdot}922 \times 10^6$	0·082
		Faeces	0·83 g.	830	46·7	387	149·5	$0{\cdot}058 \times 10^6$	0·005
		Body water	180 g.	—	—	$1{\cdot}8 \times 10^5$	0	0	0·000
		Whole blood	15·1 g.	2480	58·0	1430	36	$0{\cdot}0515 \times 10^6$	0·005
								Total	0·092
L 1 (245)	24	Urine	26 ml.	1710	43·1	737	6150	$4{\cdot}55 \times 10^6$	0·400
		Faeces	3·86 g.	3860	54·0	2080	1825	$3{\cdot}8 \times 10^6$	0·335
		Body water	176 g.	—	—	$1{\cdot}76 \times 10^5$	1·2	$0{\cdot}21 \times 10^6$	0·019
		Whole blood	14·8 g.	2740	58·0	1590	25·9	$0{\cdot}041 \times 10^6$	0·004
								Total	0·758
R 1 (220)	24	Urine	33 ml.	742	37·7	280	3340	$0{\cdot}943 \times 10^6$	0·083
		Faeces	1·77 g.	1770	51·8	920	4880	$4{\cdot}48 \times 10^6$	0·394
		Body water	173 g.	—	—	$1{\cdot}73 \times 10^5$	0·59	$0{\cdot}102 \times 10^6$	0·009
		Whole blood	14·4 g.	2640	58·0	1530	6·45	$0{\cdot}0098 \times 10^6$	0·001
								Total	0·487
B 1 (220)	32	Urine	25·5 ml.	1275	44·25	564	4750	$2{\cdot}68 \times 10^6$	0·236
		Faeces	4·31 g.	4310	46·6	2010	3650	$7{\cdot}36 \times 10^6$	0·650
		Body water	173 g.	—	—	173×10^5	0·55	$0{\cdot}09 \times 10^6$	0·008
		Whole blood	14·4 g.	2060	58·0	1200	8·55	$0{\cdot}0102 \times 10^6$	0·001
								Total	0·895
L 2 (200)	48	Urine	58 ml.	1340	45·0	602	8700	$5{\cdot}23 \times 10^6$	0·461
		Faeces	3·24 g.	3240	50·5	1630	2080	$3{\cdot}4 \times 10^6$	0·290
		Body water	176 g.	—	—	176×10^5	1·11	$0{\cdot}194 \times 10^6$	0·018
		Whole blood	14·8 g.	2150	58·0	1250	8·5	$0{\cdot}0106 \times 10^6$	0·001
								Total	0·770

Table 3. *Recovery of tritium-labelled hexoestrol from rats*

Each rat received 1 μg. of hexoestrol ($1{\cdot}136 \times 10^7$ counts/min.) in arachis oil subcutaneously. Body water was taken as 80 % and whole blood as 6·7 % of the body wt. The skin was from the site of injection.

					Combustion water from total solids				
Rat and body wt. (g.)	Time killed (hr.)	Sample	Quantity	Total solids in sample (mg.)	Yield (%)	Total (mg.)	Specific activity (counts/min./mg.)	Total activity (counts/min.)	Equivalent as hexoestrol (μg.)
P 1 (230)	8	Urine	13 ml.	198	42·0	83	$19{\cdot}7 \times 10^3$	$1{\cdot}63 \times 10^6$	0·143
		Faeces	1·45 g.	1450	64·0	926	2420	$2{\cdot}24 \times 10^6$	0·197
		Body water	184 g.	—	—	$1{\cdot}84 \times 10^5$	0·504	$0{\cdot}09 \times 10^6$	0·008
		Whole blood	15·4 g.	2910	58	1690	14·05	$0{\cdot}024 \times 10^6$	0·002
								Total	0·350
		Skin	0·151 g.	151	58	—	94	8200	0·0007
L 1 (245)	24	Urine	25 ml.	955	44·5	425	$8{\cdot}43 \times 10^3$	$3{\cdot}58 \times 10^6$	0·315
		Faeces	2·9 g.	2900	48·2	1400	3270	$4{\cdot}56 \times 10^6$	0·402
		Body water	196 g.	—	—	$1{\cdot}96 \times 10^5$	1·72	$0{\cdot}36 \times 10^6$	0·032
		Whole blood	16·4 g.	3100	58	1800	6·14	$0{\cdot}01 \times 10^6$	0·001
								Total	0·750
		Skin	0·139 g.	1·39	58	—	16·3	1 500	0·0001
R 1 (220)	24	Urine	21 ml.	1030	49·5	510	$5{\cdot}05 \times 10^3$	$2{\cdot}57 \times 10^6$	0·226
		Faeces	3·6 g.	3600	42·5	1530	6480	$9{\cdot}9 \times 10^6$	0·872
		Body water	176 g.	—	—	$1{\cdot}76 \times 10^5$	0·572	$0{\cdot}10 \times 10^6$	0·008
		Whole blood	14·7 g.	2740	58	1590	3·32	$0{\cdot}005 \times 10^6$	0·0004
								Total	1·106
		Skin	0·127 g.	127	58	—	142	10 400	0·0009
B 1 (220)	32	Urine	23 ml.	1450	38·0	550	$5{\cdot}65 \times 10^3$	$3{\cdot}11 \times 10^6$	0·274
		Faeces	4·42 g.	4420	40·8	1800	3460	$6{\cdot}22 \times 10^6$	0·546
		Body water	176 g.	—	—	$1{\cdot}76 \times 10^5$	0·446	$0{\cdot}0078 \times 10^6$	0·007
		Whole blood	14·2 g.	2781	58	1620	0·000	0·000	0·000
								Total	0·827
		Skin	0·162 g.	162	58	—	278	26 100	0·0023
L 2 (200)	48	Urine	47 ml.	1062	39·6	420	$5{\cdot}76 \times 10^3$	$2{\cdot}42 \times 10^6$	0·213
		Faeces	2·7 g.	2700	56·1	1515	4735	$7{\cdot}15 \times 10^6$	0·630
		Body water	160 g.	—	—	$1{\cdot}6 \times 10^5$	0·688	$0{\cdot}11 \times 10^6$	0·010
		Whole blood	13·4 g.	2781	58	1620	5·32	$0{\cdot}008 \times 10^6$	0·001
								Total	0·854
		Skin	0·52 g.	52	58	—	118	3 540	0·0003

animal which had received 100 μg. was assayed for tritium and found to have a specific activity too low to measure accurately, though of the order of 1 count/min./mg. Such a low specific activity was to be expected: if the whole dose had been oxidized the body water, of which the urine water may be assumed to be representative, would have acquired a specific activity of only 27 counts/min./mg. For the detection and accurate measurement of complete oxidation a much greater specific activity in the hexoestrol administered would clearly be necessary.

Rat experiments. The hexoestrol of higher specific activity was used in these experiments in the hope of detecting complete oxidation, if any. The rats were killed at different times during 48 hr. for the purpose of studying the variations, if any, in the amount of labelled hexoestrol attached to circulating blood solids and in the rate of appearance of tritium in body water. Table 2 shows the results obtained. The most important observation is the rapid rate of excretion of the drug. Even within 8 hr. (rat P 1) 8·7 % of the dose was excreted. Unfortunately, agreement between the only two replicates (L 1 and R 1, which were killed at 24 hr.) is very bad: whereas 73·5 % of the dose was excreted by L 1 only 47·7 % was excreted by R 1. The figures suggest that this low value could be due to loss of some urine from the cage in which rat R 1 was kept, although there is no record of any accident to confirm that this actually happened. If the result of rat R 1 is discarded, however, the recovery figures indicate that the bulk of the dose is excreted and that this excretion is virtually complete within 24 hr.

Table 2 also shows that the amount completely oxidized was low, although the specific activity of the body water would again be low (about 50 counts/min./mg.) even if the whole dose had been oxidized. However, the specific activity figures, which are all about 1 count/min./mg. or less, indicate that not more than 2 % of the dose was in fact completely oxidized. The specific activity of the circulating blood solids was also low, showing that less than 1 % of the dose was circulating at any time.

A second experiment (Table 3) gave results substantially in agreement with the first. It will be seen that the amount of labelled material excreted by the rat killed after 8 hr. was considerably greater (0·34 against 0·087 μg.) than was observed in the first experiment, but with one exception the recoveries at other time intervals were similar. The exception (rat R 1) showed a 110 % recovery, a result which must be wrong. It might, of course, be due to a mistake in injecting. Although every care was taken to inject exactly 0·2 ml. of arachis oil solution of labelled hexoestrol it is possible that some animals received slightly more or less than the correct amount. This could account both for the exceptionally high recovery in rat R 1 of the second experiment and for the less than quantitative recoveries in all the others. However as the amounts recovered were predominantly less than quantitative, an assay of the skin at the site of injection was carried out on all the animals of the second experiment to find out whether absorption was complete. Samples of the skin were excised, weighed, dried and assayed for tritium. As shown in Table 3 the specific activities of the combustion water from these samples ranged from 16 to 278 counts/min./mg., but corresponded to negligible quantities of labelled hexoestrol.

DISCUSSION

This work shows that even at physiological dose levels the main metabolic pathway of hexoestrol administered subcutaneously is rapid excretion in both urine and faeces. Both rabbits and two out of the ten rats excreted more in the urine than in the faeces. The rabbit which was given only 1 μg. excreted nearly equal amounts of radioactivity by these two routes, whereas that which was given 100 μg. excreted twice as much in its faeces as in its urine. This cannot, however, be interpreted as the result of differing dose levels because there was considerable variation in the relative amounts excreted by the two routes in the rat experiments, where each animal received the same dose. Leaving rat R 1 (Table 2) out of consideration for reasons already stated, it can be seen that the ratio of urinary to faecal excretion varies from 16:1 (rat P 1, Table 2) to 0·25:1 (rat R 1, Table 3). The high value of 16:1 might be due to the sample being an early one when excretion was clearly not yet complete, because in the others the scatter is not so wide, ranging from 1·6:1 (rat L 2, Table 2) to 0·25:1. No explanation of this variable excretion by the two routes is immediately obvious.

The results are, however, in general agreement with those of Twombly (1951) and of Twombly & Schoenewaldt (1951). They are also in agreement with the results of Smith & Williams (1948), who found that urinary excretion of synthetic oestrogens by rabbits was 20–40 % of the total dose after subcutaneous injections of up to 1 g. spread over several weeks. They differ, however, from the results of Hanahan *et al.* (1953) who, unlike earlier workers, found no urinary excretion of [^{14}C]-stilboestrol. These workers suggest that the apparent anomaly might be due to their having used a much smaller dose (5 μg./rat). That this is not the explanation is suggested by the present work: even at 1 μg. doses there is a substantial urinary excretion of hexoestrol or its metabolites.

It is true that Hanahan *et al.* were working with stilboestrol and not with hexoestrol but, as the excretory route is the same for both substances at high doses (Smith & Williams, 1948), one would expect it to be so at low doses, and the failure of Hanahan *et al.* to detect urinary excretion is therefore inexplicable.

Also of interest in the present work is the negligible amount of total oxidation that occurs. The amount of tritium in the body water does not amount to more than 2% of the initial dose and is probably less. This confirms that the hexoestrol was labelled in stable positions, since any labile tritium would have appeared in the body water. It agrees with the findings of Twombly (1951), Twombly & Schoenewaldt (1951) and of Hanahan *et al.* (1953), who detected no oxidation of [^{14}C]-stilboestrol. Also in agreement with the findings of these workers is ours that a negligible amount of hexoestrol is attached to circulating blood solids.

A problem that remains unresolved is the whereabouts of the 10–20% of the initial dose which is unaccounted for in the urine and faeces. Clearly it might still be in the carcasses of the animals but no analyses of tissues were made in these experiments. However, in other work (Glascock & Harris, unpublished work) it has been found that there is no accumulation of labelled hexoestrol in muscle or in any part of the nervous system, reproductive system, pituitary gland or mammary gland of spayed female rabbits killed 24 hr. after dosing with 100 μg. subcutaneously. It is of interest therefore to note that Twombly & Schoenewaldt (1951) found that, of a total recovery of 94·9% of 1·57 mg. of [^{14}C]stilboestrol injected into a mouse, only 0·17% remained in the body after 7 days. Further work is therefore necessary to discover whether or not some 10–20% of 1 μg. of hexoestrol really does remain in the body. When so large a fraction of the dose is excreted so rapidly one would not expect it to exert so profound and persistent a physiological effect unless the dose given were very much too big, the excreted fraction representing the excess. In these experiments, however, this cannot have been so: the dose of 1 μg. was at, or only slightly above, that required to produce a response. On the other hand, oestrogens of the hexoestrol type are some 200 times more active when administered locally to the vagina. Hence only a very small fraction of the dose need be retained, say in the reproductive system, for the persistence of the response after the bulk of the dose had been excreted to be more comprehensible.

SUMMARY

1. Hexoestrol generally labelled in stable positions with tritium has been injected subcutaneously in arachis oil solution at microgram dose levels into two rabbits and ten rats. One rabbit received 100 μg.; the other rabbit and the rats each received 1 μg.

2. The outstanding feature of the metabolism of the oestrogen is rapid excretion, either unchanged or as an unidentified non-volatile metabolite. Up to 90% of the initial dose was recovered in urine and faeces.

3. Excretion by both routes was substantial although the amount of radioactive material in the faeces was up to four times as much as in the urine. In some experiments, however, more was excreted in the urine than in the faeces.

4. Total oxidation as revealed by the specific activity of the body water was negligible and certainly less than 2% of the initial dose. The amount of labelled material circulating attached to the blood solids and remaining unabsorbed at the site of injection after 8 hr. or more was also negligible.

5. The results are broadly in agreement with earlier work carried out on the same and other synthetic oestrogens with very much larger doses.

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The Biosynthesis of Aromatic Compounds by *Neurospora crassa*

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The findings of Davis (1955) have shed much light on the mode of synthesis of aromatic compounds in *Escherichia coli* and other bacteria. These studies have established dehydroquinic acid, dehydroshikimic acid, shikimic acid and prephenic acid as successive precursors in the formation of the benzene ring by *E. coli*. The purpose of the present paper is to outline some of the similarities and contrasts in the synthesis of the benzene ring by *Neurospora*. Tatum (1949) and Tatum, Gross, Ehrensvaerd & Garnjobst (1954) have given evidence for the involvement of dehydroshikimic acid and shikimic acid in the metabolism of *Neurospora*, and the isolation of prephenic acid from this organism has been described (Metzenberg & Mitchell, 1956).

EXPERIMENTAL

Bacterial strains used in assays. E. coli strains 156–53M2 and 83–1, and *Aerobacter aerogenes* strain A-170-143-S1 were kindly furnished for this study by Dr B. D. Davis. The first of these strains responds only to shikimic acid as a substitute for aromatic compounds; the second can respond alternatively to shikimic acid or dehydroshikimic acid, whereas the *Aerobacter* mutant responds not only to both these compounds, but also to quinic acid and dehydroquinic acid. These strains permitted detection of any of these compounds which might be present in culture filtrates of *Neurospora* mutants, and also allowed the accumulated materials to be classified as shikimic acid, dehydroshikimic acid, or either quinic acid or dehydroquinic acid on the basis of nutritional properties for the bacterial mutants.

The medium used in these tests was that described by Davis & Mingioli (1950). Minimal medium (5 ml.) in 6 in. test tubes was supplemented with 0·5 ml. of *Neurospora* culture filtrate to be tested. The tubes were pasteurized at 70° for 15 min. and inoculated with a bacterial test strain. After incubation for 24 hr. at 35°, growth was noted by inspection. Examination of the growth of the bacterial strains with various concentrations of authentic shikimic acid revealed that the procedure used would detect 10 μg. of this compound.

Mutant strains of Neurospora. Strains 75001 and 5212, which have nutritional requirements for tryptophan and phenylalanine respectively, were available at the inception of this study and have been previously described (Bonner, 1948; Haskins, 1951). Additional mutants of *Neurospora* which require tryptophan, phenylalanine, tyrosine, *p*-aminobenzoic acid or all four of these compounds were selected by the technique of Lein, Mitchell & Houlahan (1948), and by a modification of the technique of Woodward, DeZeeuw & Srb (1954). Twenty-six strains which required the multiple aromatic supplement for growth were isolated. In addition, some 29 tryptophan-requiring strains, two phenylalanine-requiring strains and four tyrosine-requiring strains were found.

Of the 26 mutants which had multiple aromatic requirements, none showed any growth stimulation by shikimic acid, even when additionally supplemented with tyrosine and phenylalanine. Culture filtrates of 16 of these strains showed ultraviolet-absorption spectra which were indistinguishable from that of wild-type *Neurospora*. Crosses between members of this group were invariably sterile and, as a result, it has not been possible to obtain evidence for or against allelism of these strains. A typical member of this group was saved and designated as C-161.

Filtrates of the remaining 10 mutants which required a multiple aromatic supplement exhibited strong ultraviolet-absorption spectra characteristic of protocatechuic acid (3:4-dihydroxybenzoic acid). Crosses were made between several members of this group. Over 400 spores were examined and all proved to be mutant. Therefore it seems probable that these strains are allelic with respect to the altered gene. An example of this group was designated as C-163.

One of the phenylalanine-requiring strains isolated was subjected to further examination. This strain, designated as C-165, grew vigorously when supplemented with phenylpyruvic acid, and showed slow growth in the presence of tyrosine. Unlike mutant 5212 it did not accumulate prephenic acid or phenylpyruvic acid.

A tyrosine-requiring strain, C-167, has already been described in part (Metzenberg & Mitchell, 1956). Crosses were made between this strain and each of the other tyrosine-requiring strains. Between 300 and 700 spores from each cross were examined and in no case were any wild types found. These four strains have therefore been assumed to be allelic.

RESULTS

Evidence that the observed metabolic requirements of the Neurospora *strains is due to a single gene alteration in each case.* Each of the mutants was crossed to wild-type *Neurospora* of the opposite mating type on slants of medium (Westergaard & Mitchell, 1947) supplemented with DL-tryptophan, DL-phenylalanine, L-tyrosine (each at 100 μg./ml.) and *p*-aminobenzoic acid (0·5 μg./ml.). The crosses were incubated until ripe, the asci then dissected

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and the spores induced to germinate by heat treatment on plates of minimal agar. The germinated spores could easily be classified as mutant or wild type by the length of the hyphae after growth for 12 hr. The results are given in Table 1. It will be seen that in each case, the data are consistent with the interpretation that the observed nutritional requirements are due to a single gene alteration.

Preparation of Neurospora *strains containing more than one mutant gene.* Strains C-161 and C-163 of opposite mating types were crossed, asci dissected and the separated spores induced to germinate. One ascus was found to contain two mutant spore pairs and two wild-type spore pairs. Each of the mutant spores may therefore be assumed to contain both the C-161 and C-163 genes. This assumption was confirmed by crossing one of the presumed double-mutant strains to wild type and observing several asci containing more than two mutant spore pairs. This double mutant will be referred to as C-161/C-163.

The phenylalanine-requiring strains C-165 and 5212 were crossed and a double mutant was obtained. The genetic constitution of the double mutant C-165/5212 was established in the same manner as with C-161/C-163.

The multiple mutants C-165/C-167, 75001/C-165, 75001/C-167, 75001/C-165/C-167 and 75001/5212/C-167 were obtained from crosses in a similar manner. In each case the nutritional requirements were those to be expected from the component genes, and therefore it was not felt necessary to confirm the genotypes of these multiple strains by genetic analysis. The triple mutant 75001/5212/C-167 has previously been described with respect to accumulation of prephenic acid (Metzenberg & Mitchell, 1956).

Table 1. *Evidence that nutritional requirements of* Neurospora *mutants are due to single gene alterations*

Neurospora strain	No. of asci observed	Asci containing more than two mutant spore pairs	Asci showing second-division segregation
C-161	29	0	5
C-163	14	0	1
C-165	9	0	3
C-167	26	0	20

Order of the series of Neurospora *mutants in the biosynthetic pathway.* No cross-feeding behaviour could be detected between C-161 and C-163, the two mutants which exhibit a multiple aromatic requirement for growth. This situation is in contrast with that described by Davis in *E. coli*. It has been mentioned that C-163 accumulates a compound with the absorption spectrum of protocatechuic acid, and isolation and identification of this material is presented in a later section of this paper. The catabolism of this compound has been studied in detail by Gross, Gafford & Tatum (1956). It was found that the double mutant C-161/C-163 accumulates no protocatechuic acid and thus resembles C-161. Therefore it appears that the metabolic lesion associated with C-161 occurs earlier in the biosynthetic sequence than that associated with C-163. This technique of establishing the order of a series of mutant genes in a pathway has been used previously (Mitchell & Houlahan, 1946; Haas, Mitchell, Ames & Mitchell, 1952).

The phenylalanine-requiring strains C-165 and 5212 may be placed in order by the observation that both strains readily utilize phenylpyruvic acid as a substitute for phenylalanine. It has been shown (Metzenberg & Mitchell, 1956) that 5212 excretes prephenic acid, which is converted into phenylpyruvic acid by the acidity of the media usually used for culture of *Neurospora*. Since 5212 apparently lacks prephenic decarboxylase, and C-165 apparently does *not* lack the transaminase required to convert phenylpyruvic acid into phenylalanine, C-165 must represent an earlier lesion in the pathway to phenylalanine than does 5212. This conclusion is corroborated by the behaviour of the double mutant C-165/5212, which accumulates neither prephenic acid nor phenylpyruvic acid and thus resembles C-165. The order of metabolic lesions concluded from these data is presented in Fig. 1.

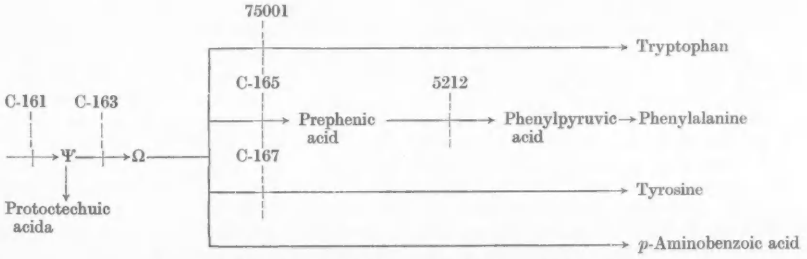

Fig. 1. Order of metabolic lesions in the biosynthetic sequence.

It is apparent that the triple-blocked strain 75001/C-165/C-167 might well accumulate the last precursor (i.e., the 'branch-point' compound Ω), which is common to the three aromatic amino acids and *p*-aminobenzoic acid. Such a compound would perhaps be difficult to demonstrate by studies of an organism in which genetic manipulation is not feasible. It was found that this triple-blocked strain accumulated no material which would satisfy all or any of the nutritional requirements of C-161; however, this strain did accumulate protocatechuic acid, vanillic acid and dehydroshikimic acid. Evidence for the presence of these compounds in culture filtrates of *Neurospora* mutants is presented below.

Identification of materials accumulated by mutant strains

Protocatechuic acid. Erlenmeyer flasks (125 ml.) were charged with 20 ml. each of Fries medium (Fries, 1938) supplemented with DL-tryptophan, DL-phenylalanine and L-tyrosine, each at a concentration of 5 μg./ml., and *p*-aminobenzoic acid (0·1 μg./ml.). The flasks were inoculated with conidia of C-163. After 5 days' growth at 25°, the culture filtrates were pooled and agitated with sufficient Norit to remove the characteristic ultraviolet absorption (approx. 3 g./l.). The Norit was treated with boiling ethanol in an atmosphere of nitrogen for 10 min. The ethanolic eluate was evaporated *in vacuo*. The residue was taken up in a little water and extracted with ligroin. Evaporation of the latter gave white crystals, which were further purified by sublimation onto a solid carbon dioxide 'cold finger' *in vacuo*.

The material was recrystallized from water. The isolated protocatechuic acid had m.p. 201–202°, unchanged by admixture with an authentic sample (Found: C, 54·9; H, 4·3. $C_7H_6O_4$ requires C, 54·6; H, 3·9%). Melting points were uncorrected.

The ultraviolet-absorption spectrum of the isolated protocatechuic acid was found to be identical with that of an authentic sample. Chromatographic comparison of these in three solvent systems [propanol–aq. M-NH_3 soln. (3:1, v/v); propanol–aq. M-acetic acid (3:1, v/v); butanol–ethanol–water (4:1:1, by vol.)] and detection by spraying with aq. ferric chloride (1%, w/v) revealed no differences.

Culture filtrates of the triple-blocked strain 75001/C-165/C-167 were prepared in the following manner. The strain was grown in a carboy containing 15 l. of the medium of Westergaard & Mitchell (1947) supplemented with 25 μg./ml. each of DL-tryptophan, DL-phenylalanine and L-tyrosine. Protocatechuic acid was isolated essentially as described above. The material was characterized as before by absorption spectrum, melting point and chromatographic behaviour.

Vanillic acid. During the course of isolation of protocatechuic acid from 75001/C-165/C-167, the mother liquors from crystallization of the above compound were examined chromatographically for the presence of other compounds. In a solvent system consisting of 2-methylpropan-2-ol–aq. M-NH_3 soln. (30:10, v/v) two compounds which migrated more rapidly than protocatechuic acid were revealed by spraying the chromatogram with diazotized sulphanilic acid (Ames & Mitchell, 1952). The material of intermediate mobility, which was present in considerable amounts, gave an orange colour with this reagent. The liquors were applied to strips of Schleicher and Schüll no. 470 paper (Carl Schleicher und Schüll, Dassel, Germany), and the chromatogram was developed by the ascending method with the solvent system mentioned above. Guide strips were cut and the position of the material of intermediate mobility was determined by spraying with diazotized sulphanilic acid. The remainder of the material was then eluted with water and the eluate evaporated to dryness. The material was further purified by vacuum sublimation in a temperature-gradient tube. The properties of the compound did not appear to be changed by this treatment. The resulting material was recrystallized six times from dilute 2-methylpropan-2-ol and once from ligroin–*n*-propanol. The neutralization equivalent was found to be 172, in reasonably good agreement with the theoretical value of 168. The ultraviolet-absorption spectra in acid and in alkali were identical with those of vanillic acid synthesized by the method of Pearl (1946). The chromatographic behaviour of the two preparations was identical in five solvent systems [butanol–ethanol–water (4:1:1, by vol.); propanol–pyridine–benzene–water (3:1:1:1, by vol.); 2-methylpropan-2-ol–aq. M-NH_3 soln. (3:1, v/v); 2-methylpropan-2-ol–aq. M-NH_3 soln.–pyridine–benzene–water (6:1:1:1:1, by vol.); 2-methylpropan-2-ol–pyridine–water (6:1:1, by vol.)]. The sample isolated had m.p. 205–206°. The mixed m.p. with synthetic vanillic acid (m.p. 207–208°) was 205–207°.

Dehydroshikimic acid. Culture filtrates of 75001/C-165/C-167 were examined for the presence of materials which would stimulate growth of the bacterial strains. It was found that these filtrates promoted rapid growth of strains 83-1 and A-170-143-S 1, but no growth of 156–53M2. On these grounds, it was suspected that dehydroshikimic acid was present in filtrates of this triple-blocked mutant. In order to obtain further evidence for the nature of the material, filtrate (50 ml.) from the carboy from which protocatechuic acid and vanillic acid were isolated was evaporated to dryness and

redissolved in water (5 ml.). Methanol (50 ml.) was added and the precipitate of inorganic salts removed by centrifuging. The supernatant liquid was evaporated and the residue was dissolved in water (1 ml.).

For purposes of comparison, a concentrate containing dehydroshikimic acid was prepared from culture filtrates of *E. coli* strain 83–2, which is known to accumulate the latter compound. Erlenmeyer flasks (125 ml.) were charged with 40 ml. of the medium recommended by Salamon & Davis (1953). The flasks were inoculated and incubated at 35° for 2 days with gentle agitation. The cells were removed by centrifuging and the supernatant medium was prepared for chromatography as described above.

The desalted preparation from the triple-blocked *Neurospora* mutant and the dehydroshikimic acid preparation from the *E. coli* mutant were subjected to paper chromatography in three solvent systems. These solvents were: *A*, ethanol–pentan-1-ol–M-acetic acid (2:1:1, by vol.); *B*, 2-methylpropan-2-ol–M-acetic acid (3:1, v/v); *C*, 2-methylpropan-2-ol–88% formic acid–water (2:1:1, by vol.).

Shikimic acid was also applied to chromatograms for purposes of comparison. After development, the chromatograms were cut horizontally into ten equal sections, and the sections eluted with 2 ml. of the medium of Davis & Mingioli (1950), and supplemented with DL-phenylalanine and L-tyrosine (each 25 μg./ml.) to increase the sensitivity of the assay. The tubes were pasteurized as before and inoculated with *Aerobacter aerogenes* strain A-170-143-S 1. On examination of the assay tubes from the chromatograms, it was apparent that the biologically active material in the *Neurospora* culture filtrate was chromatographically identical with dehydroshikimic acid. The results are presented in Table 2. It will be apparent that the second decimal place given for each R_F value is of only relative significance.

The *Neurospora* strains described in this paper were examined for the presence of biologically active materials by use of the bacterial-assay strains. The results are given in Table 3.

Table 2. *Chromatographic properties of shikimic acid and dehydroshikimic acid from* Escherichia coli, *and biologically active material from* Neurospora *strain* 75001/*C*-165/*C*-167

	R_F		
	Solvent *A*	Solvent *B*	Solvent *C*
Shikimic acid	0·65	0·45	0·75
Dehydroshikimic acid	0·40	0·05–0·1	0·05–0·1
Neurospora material	0·40	0·05–0·1	0·05–0·1

Table 3. *Biologically active materials accumulated by* Neurospora *mutants*

Degree of growth is indicated by the symbols + +, approx. 10^8 bacteria/ml.; +, approx. 10^7 bacteria/ml.; ±approx. 10^6 bacteria/ml.; −, fewer than 10^6 bacteria/ml.

Culture filtrate from *Neurospora* strain	Growth of bacterial strains		
	156–53 M-2	83-1	A-170-143-S 1
C-161	−	−	−
C-163	−	±	±
75001	−	−	−
C-165	−	−	−
5212	−	−	−
C-167	−	−	−
C-165/5212	−	+ +	+ +
C-165/C-167	−	+ +	+ +
75001/C-165	−	−	−
75001/C-167	−	−	−
75001/C-165/C-167	−	+ +	+ +
75001/5212/C-167	−	+	+

DISCUSSION

It will be seen from Table 3 that, except for C-163, which possibly accumulates a biologically active material at the very borderline of detection, none of the *Neurospora* strains examined containing a single metabolic lesion has been found to excrete shikimic acid, dehydroshikimic acid, dehydroquinic acid or quinic acid. On the other hand, a number of the multiple mutants accumulate material with the nutritional characteristics of dehydroshikimic acid. It is also to be noted that shikimic acid is not accumulated by these strains. However, the data presented here should not be taken as evidence against the participation of shikimic acid in the biosynthesis of the aromatic compounds, as the strains examined may be impermeable to shikimic acid. The provisional pathway outlined in Fig. 1 suggests the hypothesis that the 'branch-point' compound is not accumulated by a singly blocked mutant, e.g. C-165, for the reason that it is diverted into the production of tryptophan or tyrosine; in the strains with multiple blocks this diversion does not occur, and the compound is accumulated. The presence of biologically active material in filtrates of C-165/5212 is somewhat anomalous and remains to be fully explained. However, it is tempting to speculate from the behaviour of the *Neurospora* mutants that dehydroshikimic acid or a closely related compound may be the 'branch-point' compound Ω in *Neurospora*, rather than being a relatively early precursor of aromatic compounds, as with *E. coli*.

The origin of protocatechuic acid and vanillic acid deserves some comment. These compounds are at the oxidation level of dehydroshikimic acid, and are probably detoxification products of the latter. Neither compound promotes the growth of any of

the *Neurospora* or bacterial mutants. In addition, it seems improbable on structural grounds that these benzenoid compounds would be intermediates in the synthesis of such hydroaromatic compounds as prephenic acid. It is not known at present whether vanillic acid is formed by methylation of the 3-hydroxyl group of dehydroshikimic acid followed by dehydration, or by direct methylation of protocatechuic acid. Gross *et al.* (1956) have shown that vanillic acid is a potent inducer of protocatechuic acid oxidase in *Neurospora*, and therefore it is of added interest that this compound occurs naturally in *Neurospora*.

SUMMARY

1. Some strains of *Neurospora* showing a nutritional requirement for aromatic compounds have been obtained. Preparation of multiple mutants is described.

2. Evidence for the presence of protocatechuic acid, vanillic acid and dehydroshikimic acid in culture filtrates of some of these mutants is presented.

3. The possibility of a central role for dehydroshikimic acid in the synthesis of aromatic compounds by *Neurospora* is discussed.

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Enzymic Determination of Acetaldehyde in Blood

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The determination of acetaldehyde in blood is of interest in the study of ethanol metabolism, where this substance may be expected to occur as an intermediate, especially during treatment with substances such as tetraethyl thiuram disulphide, [bis(diethylthiocarbamoyl) disulphide], which presumably interfere with aldehyde breakdown. The methods available are, however, not entirely satisfactory. Among the purely chemical methods the most reliable is undoubtedly that of Stotz (1943) based on the colour reaction with *p*-hydroxydiphenyl in strong sulphuric acid. The procedure requires, however, distillation involving special apparatus, and is very sensitive to trace contamination. Moreover, interference by various normal metabolic products cannot be excluded. The elegant method of Burridge, Hine & Schick (1950) involves the use of Conway micro-diffusion units. Acetaldehyde is caught in semicarbazide in a buffer solution and the light absorption of the semicarbazone at 224 mμ is used for the estimation. Unfortunately this method, though very sensitive, is not sufficiently specific. Acetoacetate in the blood will give rise to acetone under the analytical conditions, and this substance interferes in the measurement (see below).

Enzymic methods for determination of acetaldehyde have also been suggested (Holzer, Holzer & Schultz, 1955; Racker, 1957). The oxidation of reduced diphosphopyridine nucleotide (DPN) by aldehydes, catalysed by yeast alcohol dehydrogenase, may be used quantitatively, although the

method is not very accurate at low levels of aldehyde because it involves the measurement of a small decrease in a rather large optical density. Moreover, the reaction with aldehyde is not quantitative when large amounts of alcohol are present, because the equilibrium is changed towards formation of aldehyde. This method has been employed by Brahm-Vogelsanger & Wagner (1957).

Aldehyde dehydrogenase from ox liver (Racker, 1949) would appear to be a very suitable enzyme because the equilibrium is totally in favour of acetate and the enzyme has a very small Michaelis constant towards aldehyde. The reduction of DPN, even by extremely small amounts of aldehyde, consequently proceeds stoicheiometrically within a short time. There are, however, certain disadvantages in this method, which we have tried extensively for the determination of acetaldehyde in liver homogenates. The first is that the enzyme is rather difficult to prepare and not very stable. The second and major disadvantage is the presence of lactic and malic dehydrogenases in the enzyme preparations made according to Racker (1949). Several attempts to remove these enzymes, which interfere badly in the analysis of tissue extracts containing lactate and other intermediary metabolites, were unsuccessful and consequently we looked for another source of aldehyde dehydrogenase. Black (1951) has purified a potassium-requiring enzyme from baker's yeast, which catalyses the dehydrogenation of acetaldehyde to acetic acid with either DPN or triphosphopyridine nucleotide (TPN) as coenzyme. This enzyme is very easy to prepare in a state of sufficient purity. The preparations contain no DPN-requiring lactic dehydrogenase or other interfering enzymes and are stable for several months at $-20°$.

The kinetic properties of the enzyme are very different from those of aldehyde dehydrogenase from liver. The affinity between aldehyde and enzyme is very small, and even though the equilibrium is situated far towards acetate, acetaldehyde is not quantitatively oxidized to acetate. The rate of the reaction is, however, proportional to the aldehyde concentration within rather wide limits and is employed for the quantitative determination of aldehydes. The specificity of Black's aldehyde dehydrogenase is similar to that of alcohol dehydrogenase and liver aldehyde dehydrogenase and is thus far from ideal, as a number of aliphatic and even aromatic aldehydes are attacked. For the study of alcohol–acetaldehyde metabolism this lack of strict specificity is, however, of no importance.

EXPERIMENTAL

Procedure for aldehyde determination in blood plasma by the diffusion technique. As the mixing of reagents and blood plasma in the Conway units as performed by Burridge *et al.* (1950) proved difficult, the procedure was slightly modified. Plasma, 0·67 N-H_2SO_4 and 10% (w/v) sodium tungstate are mixed thoroughly in glass-stoppered centrifuge tubes in the cold and 5 ml. of the mixture is transferred to the Conway unit. The rest of the procedure is in accordance with Burridge *et al.*

Preparation of yeast aldehyde dehydrogenase. Our procedure differs in some respects from that of Black (1951, 1955). The starting material is commercial dry yeast (Danisco). This yields much less enzyme than fresh baker's yeast, but does not require the use of liquid air. Dry yeast (100 g.) is extracted at room temperature with 500 ml. of 0·3 M-K_2HPO_4 with mechanical stirring for 2 hr., while the pH is kept at 8·6 by addition of conc. aq. NH_3 soln. The suspension is kept at 4° overnight and centrifuged at 2000 *g* for 25 min. The nearly clear supernatant is treated as described by Black (1951, 1955). Before collection of the precipitate, formed in step 3 by addition of acid to pH 5, the acidified solution is kept for 2 hr. at 4°. The precipitate is dissolved as described by Black, and the solution is reacidified to pH 5. The precipitate is collected by centrifuging and dissolved in approx. 10 ml. of buffer containing 0·025 M-K_2HPO_4 and 0·001 M-cysteine hydrochloride. The pH is adjusted to 6·3 and traces of turbidity are removed by centrifuging. This reprecipitation is performed to remove traces of alcohol dehydrogenase. The enzymic activity of the final solution is measured according to Black. It is regularly found to contain about 200 000 units of enzyme. Alcohol dehydrogenase is measured according to Racker (1950), except that the buffer contains 0·07 M-semicarbazide. The optical density at 340 mμ is followed for 10 min. The increase should be less than 0·01 for 25 μl. of enzyme. The enzyme preparation is distributed in small tubes containing approx. 10 000 units each and kept at $-20°$. It keeps well for several months under these conditions.

Determination of acetaldehyde in blood plasma

Metaphosphoric acid (0·75 M). Sticks (12 g.) of metaphosphoric acid (Merck) are powdered in a mortar and dissolved in 100 ml. of water. This product contains a considerable amount of $Na_4P_2O_7$ added to make the product hard. Pyrophosphate does not interfere unfavourably in the analyses. The solution is kept well-stoppered in the refrigerator, but even at 0° a fairly rapid hydrolysis takes place. The solution is titrated before use and should be discarded when more than a week old.

Potassium hydroxide (1·25 M). A volume (0·5 ml.) of this solution is used to neutralize 2 ml. of deproteinized blood plasma. If the metaphosphoric acid solution is partially hydrolysed it is convenient to dilute a 1·5 N-KOH stock solution according to the titre of the metaphosphoric acid so that 0·5 ml. will neutralize 2 ml. of plasma filtrate.

DPN–buffer mixture. This is prepared by mixing M-KCl (3 vol.), M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 8·0, (6 vol.), 0·1 M-ethylenediaminetetraacetate (0·5 vol.), 0·1 M-mercaptoethanol (0·5 vol.) and 0·015 M-DPN (0·5 vol.).

Stock solution of acetaldehyde (2 M). Freshly distilled acetaldehyde is diluted with approx. 9 vol. of water. The concentration is determined by titration. This solution keeps well in the refrigerator, but should be checked by titration every second month.

All chemicals used were of analytical grade. DPN was the product of C. F. Boehringer und Soehne.

Deproteinization. Blood is collected in plastic centrifuge tubes, which are immediately placed in ice and closed with plastic stoppers. The blood is centrifuged at 4° at 2000 *g* for 10 min. No clotting is observed under these conditions. Plasma (2 ml.) is measured into glass-stoppered 8 ml. centrifuge tubes containing 2 ml. of ice-cold 0·75 M-metaphosphoric acid. After thorough mixing and centrifuging, 2 ml. of the clear supernatant is transferred (by a semi-automatic Carlsberg pipette) to cold, stoppered tubes and neutralized by 0·5 ml. of KOH solution (1·25 M). At this stage the samples may be left in the refrigerator overnight without significant loss of acetaldehyde.

Measurement. The neutralized filtrate (2 ml.) is mixed with 1 ml. of the DPN–buffer mixture and placed in 3 ml. cuvettes in a Hilger Uvispek spectrophotometer (light path 1 cm.). The optical density at 340 mμ is measured and 25 μl. or a volume containing at least 250 units of yeast aldehyde dehydrogenase is added with simultaneous stirring. The optical density is measured again after the lapse of 10 min. A polyethylene lid on the cuvettes prevents evaporation of aldehyde during the measurement. For each series of analyses blank and standard solutions are included. The blank analysis is made with metaphosphoric acid diluted 1:1 and neutralized with KOH. The standard is made by addition to this solution of 1–10 μg. of acetaldehyde (25 μl. of a suitably diluted stock solution). The increase in optical density in 10 min. of the blank analysis is in general around 0·01, provided that the reagents contain no trace of aldehyde. This value is subtracted from all other readings and the concentration of aldehyde calculated from the proportionality between concentration and increase in optical density (see Fig. 1). It is important to keep the temperature below 15° to limit evaporation of aldehyde.

RESULTS

Diffusion method. In a series of ten analyses of the same plasma with acetaldehyde added to a concentration of 9 μg./ml., the optical density (±S.D.) at 224 mμ was 0·334±0·011. Acetone added to blood plasma gave an optical density of 0·12 for 10 μg. of acetone/ml. of plasma. Acetone itself may not normally be present in blood to any measurable extent, but the possibility that acetoacetate under the conditions of the analysis may give rise to acetone was examined. A sodium acetoacetate solution, prepared by alkaline hydrolysis of the ethyl ester (Seeley, 1955), was concentrated *in vacuo* at low temperature to remove traces of free acetone. Under the conditions used for analysis of plasma it was found that 2–5% of the acetoacetate was transformed into acetone, thus giving rise to a significant error. A concentration of acetoacetate in blood plasma about 10 mg./100 ml. will result in an optical density corresponding to about 1 μg of acetaldehyde/ml. The magnitude of this error in analyses of blood from a normal individual is illustrated in Table 1.

Table 1. *Comparison of diffusion method and enzymic method for the determination of acetaldehyde in blood plasma*

Four blood samples were taken from a subject fasted for 12 hr., at intervals of approx. 1 hr. All analyses were made in duplicate.

Sample no.	Acetaldehyde in plasma (μg./ml.) Diffusion method	Enzymic method
1	0·9	0·1
2	1·0	0·1
3	1·0	0·1
4	1·0	0·1

Optimum conditions for the enzymic determination of acetaldehyde

Deproteinization. A number of different methods for deproteinization have been tried. Trichloroacetic acid inhibited the yeast enzyme by about 50%, and perchloric acid interfered seriously with the determination. Analysis of blood plasma could be performed after zinc or cadmium deproteinization, but very slight deviations from the correct pH after addition of the reagents (zinc sulphate and potassium hydroxide) led to low recoveries or inhibition of the enzyme. Metaphosphoric acid was found, however, to be applicable for deproteinization both of blood plasma and tissue homogenates.

Activity of the enzyme. The rate of the reaction between DPN and acetaldehyde is proportional to the concentration of aldehyde up to 0·1 mM. It is convenient to use an interval of 10 min. for the measurement. This gives an increase in absorption corresponding to approximately half conversion into acetate. When the increase in optical density

Fig. 1. Relation between concentration of acetaldehyde (μg. of acetaldehyde in the cuvette) and the increase in optical density at 340 mμ in 10 min. The aldehyde was dissolved in 2 ml. of neutralized metaphosphoric acid + 1 ml. of DPN–buffer mixture and 25 μl. of enzyme was added. The points represent average values of two determinations.

Table 2. *Reaction of different aldehydes with yeast aldehyde dehydrogenase*

Aldehyde solution (25 μl.) was added to 3 ml. of neutralized metaphosphoric acid + DPN–buffer mixture. The aldehydes, except trichloroacetaldehyde and glyceraldehyde, were freshly distilled and showed the correct boiling point.

	Concn. (mM)	Increase in optical density at 340 mμ	Percentage of the value for acetaldehyde*
Acetaldehyde	5·3	0·148	100
Formaldehyde	5·0	0	0
Propionaldehyde	4·85	0·151	111
n-Butyraldehyde	4·83	0·107	79
*iso*Butyraldehyde	4·86	0·056	41
Crotonaldehyde	5·34	0·160	107
Trichloroacetaldehyde	4·90	0	0
DL-Glyceraldehyde	4·80	0	0
Benzaldehyde	5·28	0·179	121
Salicylaldehyde	5·20	0	0
Phenylacetaldehyde	5	0·033	24

* The optical densities were corrected to the same concentration.

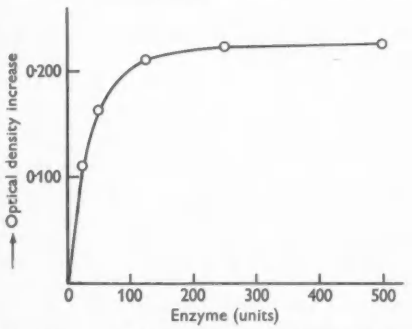

Fig. 2. Influence of the concentration of yeast aldehyde dehydrogenase on the reaction rate expressed as increase in optical density at 340 mμ in 10 min. The reaction mixture contained 7·4 μg. of acetaldehyde in 2 ml. of neutralized metaphosphoric acid + 1 ml. of DPN–buffer mixture. The measurements were corrected for variation in blank value caused by variations in the amount of enzyme preparation added. The points are average values of two determinations.

under our analytical conditions is plotted against concentration of aldehyde, proportionality is found within a quite satisfactory range (see Fig. 1).

When the concentration of enzyme is increased above a certain level the reaction rate becomes approximately constant (Fig. 2). We find it convenient to employ 250 units for each measurement. In this way reproducible results are found and the influence of small amounts of inhibitory substances is eliminated.

Hydrogen-ion concentration. A sufficient amount of tris buffer is used to ensure that the pH is near 8 in the final reaction mixture, if the metaphosphoric acid filtrates have been neutralized as described in the Experimental section. The reaction proceeds somewhat faster at more alkaline reaction, but as the enzyme is more stable at or below pH 8 we have found this to give better results.

Table 3. *Recovery of acetaldehyde added to blood plasma*

Acetaldehyde added (μg./ml.)	Acetaldehyde found (μg./ml.)	Recovery (%)
0	0·2	—
2·32	2·52	100
2·32	2·52	100
2·32	2·70	108
4·62	4·73	98
4·62	4·73	98
4·62	4·66	97

Specificity of the method. A number of aldehydes react with yeast aldehyde dehydrogenase under the conditions used for analysis of blood and tissue. Table 2 presents a summary of the results.

Recovery of acetaldehyde added to blood. It is important to keep blood samples cold and well-stoppered to prevent loss of acetaldehyde by evaporation. Separation of plasma should be done as soon as possible, as aldehyde seems to disappear more rapidly from whole blood than from plasma (Stotz, 1943). Acetaldehyde added to plasma or serum was quantitatively recovered when deproteinization was performed with metaphosphoric acid, and at a level of acetaldehyde as low as 1 μg./ml. Table 3 shows the recovery of acetaldehyde added to blood plasma.

Accuracy and sensitivity. An impression of the accuracy of the method was obtained through independent analyses of one plasma sample. For each analysis 2 ml. of the plasma was precipitated with metaphosphoric acid. For eleven samples containing 4·6 μg./ml. of acetaldehyde the increase in optical density $\pm$ S.D. in 10 min. was 0·101 $\pm$ 0·002. The sensitivity of the method is dependent on the stability of the spectrophotometer employed. We

have consistently been able to detect an increase in optical density of 0·002 above the reagent blank. This corresponds to a limit for the detection of acetaldehyde in plasma of approx. 0·1 μg./ml.

DISCUSSION

As seen in Table 2 the method will measure a number of aldehydes besides acetaldehyde. None of these is, however, believed to occur in animals. It should nevertheless be kept in mind that the concentrations found by the enzymic method represent maximum values, even though they are considerably lower (Lundquist, F. & Wolthers, H. unpublished work) than those observed by the chemical methods (Raby, 1954).

The presence of enzymes other than aldehyde dehydrogenase in the preparation used may result in errors. Alcohol dehydrogenase would cause too high results when alcohol is present. As estimation of aldehyde is of interest primarily in connexion with alcohol metabolism, it is, of course, important to avoid this error. With the procedure described for the purification of the enzyme, alcohol dehydrogenase is completely removed. Numerous experiments have been performed with high concentration of ethanol in biological material and the absence of interference in the analytical method has been established. In order to demonstrate this lack of interference it proved necessary to purify the ethanol by distillation over metal sodium to remove traces of aldehyde. Ethanol thus purified and added to blood plasma in amounts from 0·1 to 2% had no influence on the measurement. Addition of acetate to plasma up to 0·3M is likewise without any influence on the measurement of acetaldehyde.

Other DPN-dependent enzymes may contaminate the preparation employed and could attack substrates present in deproteinized filtrates from biological material. This possibility cannot be excluded completely, but the virtual absence of increase in optical density on examination of liver homogenates, which presumably contain a number of intermediary metabolites, shows that this source of error is of no practical importance.

The concentration of acetaldehyde measured in blood plasma from normal subjects during metabolism of ethanol is extremely low (about 0·2 μg./ml., Lundquist, F. & Wolthers, H. unpublished work), and near the limit of detection for the present method. It would therefore be most desirable to be able to increase the sensitivity. Various possibilities have been considered without much success so far. Theorell, Nygaard & Bonnichsen (1955) have employed the fluorescence of reduced DPN to determine very small concentrations of this substance. Preliminary experiments showed, however, that the fluoresence of protein-free filtrates from blood and tissues is so large that the increased sensitivity of the measurement of reduced DPN is completely compromised.

Another possibility is to measure the DPN formed from reduced DPN when alcohol dehydrogenase is used. DPN, under the conditions described by Robinson, Levitas, Rosen & Perlzweig (1947) and Carpenter & Kodicek (1950), gives a fairly strong fluorescence which may be used for quantitative measurement. Preliminary experiments made with this method were, however, disappointing. It appeared that the presence of a large excess of reduced DPN inhibits the reaction so that the overall sensitivity is not better than that of the aldehyde dehydrogenase method.

If the fluoresence method were combined with a distillation of the material to be analysed, it should be possible to increase the sensitivity some ten times, but so far no experiments in this direction have been performed.

SUMMARY

1. The determination of acetaldehyde in blood by diffusion into semicarbazide is subject to interference from acetoacetate, which is partly decarboxylated to acetone during the procedure.

2. A method is described based on the use of a potassium-requiring aldehyde dehydrogenase easily prepared from commercial dry yeast. The rate of formation of reduced diphosphopyridine nucleotide under standard conditions is proportional to the aldehyde concentration within a considerable range.

3. Under the analytical conditions chosen a number of aldehydes will react; the specificity is therefore not complete.

4. The accuracy of the method at very low aldehyde concentrations is better than that obtainable with other methods. The limit for detection of acetaldehyde in biological material is around 0·1 μg./g.

This investigation has been partially supported through a grant from Statens almindelige Videnskabsfond. I am indebted to Miss Helen Pedersen for careful technical assistance. The continued interest which Professor Dr Knud Sand has shown in this investigation is gratefully acknowledged.

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The Colorimetric Determination of Dipeptides by means of Oxygenated Cobalt Complexes

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Numerous methods have been proposed for the determination of dipeptide hydrolysis (Martin & Synge, 1945), but no single one of them satisfies the dual requirements of rapidity and accuracy. Procedures based on acid–base titration lack precision because of the large blank due to the substrate; this is true also of the colorimetric–ninhydrin (Schwartz & Engel, 1950) and ammonia–ninhydrin (MacFadyen, 1944) methods. Measurement of the carbon dioxide evolved in the ninhydrin reaction has been made the basis of quantitative procedures (Van Slyke, MacFadyen & Hamilton, 1941; Van Slyke, Dillon, MacFadyen & Hamilton, 1941); these have the advantages that, since simple dipeptides evolve no carbon dioxide, amino acids are specifically determined. A disadvantage of these methods is the laborious and time-consuming technique required. Methods based on the formation of copper complexes (Spies & Chambers, 1951; Spies, 1952) are imprecise because, even under the most favourable conditions, dipeptides give 70–80% of the extinction produced by their constituent amino acids. The Van Slyke nitrous acid technique has many disavantages, notably the time required per determination and the non-stoicheiometric reaction.

In view of these difficulties it was considered desirable to explore the possibility of utilizing other reactions for analytical purposes.

Smith (1948) observed that, when glycylglycine was incubated for several hours at room temperature and pH 8 with Co^{2+} ions, a pink colour developed which was more intense than the colour produced by an equivalent quantity of glycine. The reaction is not restricted to glycylglycine and similar colour production from other dipeptides has been observed. Gilbert, Otey & Price (1951) made the significant discovery that atmospheric oxygen is involved in the colour-forming reaction and that the coloured compound is an oxygen-containing cobaltous complex. Suitable conditions have been found for the utilization of this reaction as a quantitative analytical method by using hydrogen peroxide as oxygenating agent and a suspension of cobaltous phosphate in borate buffer as the source of cobaltous ions (Crook & Rabin, 1954). Techniques have been evolved for the determination of dipeptide hydrolysis at two levels: these are designated the 'macro' and 'micro' methods and the choice of either depends on the availability and cost of the substrates.

MATERIALS AND METHODS

Glycyl-DL-alanine, glycyl-L-tryptophan, DL-alanyl-DL-alanine, glycyl-DL-phenylalanine, glycyl-L-tyrosine and acetylglycine were obtained from L. Light and Co., Colnbrook, Bucks.

Glycylglycine, glycyl-DL-leucine, DL-alanylglycine, DL-leucylglycine and diglycylglycine were obtained from Roche Products Ltd., Welwyn Garden City, Herts.

Sarcosylglycine and *N*-ethylglycylglycine were prepared by the method of Levene, Simms & Pfaltz (1924) and reprecipitated twice by the addition of ethanol to an aqueous solution.

Glycyl-L-proline was prepared by the method of Bergmann, Zervas, Schleich & Leimert (1932). The amino acids used were commercial preparations available in this laboratory.

Absorption spectra were measured in a Unicam Model SP. 500 quartz spectrophotometer with cells of 1 cm. path length.

Colorimetric determinations were made in a Gallenkamp Direct-Reading colorimeter with cells of 1 cm. path length.

Preparation of suspension of cobaltous phosphate in borate buffer (pH 9·1). The suspension is prepared by adding 3 vol. of 0·2 M-cobaltous chloride solution to 2 vol. of 0·2 M-trisodium phosphate solution, mixing thoroughly and diluting with 10 vol. of 0·1 M-borax solution. The suspension should be shaken well before use; it can be used immediately but keeps for several weeks after preparation.

'*Macro*' *method*. The sample, placed in a flask graduated at 25 ml., should contain approx. 80 μmoles of dipeptide or hydrolysis products. The sample may have any volume up to 10 ml. without significantly affecting the colour development. Ethanolic thymolphthalein (0·1%, 3 drops) is added and the solution made blue by the addition of 0·5 N-NaOH. The cobaltous phosphate suspension is then added from a pipette with a wide tip, and the solution placed in a boiling-water bath. At the end of each of three successive 10 min. intervals, hydrogen peroxide (0·5 ml. of '2 volumes', AnalaR or equivalent) is added slowly with constant shaking. The flask is removed from the bath for the addition of the peroxide and replaced when the addition is completed. After the final addition of peroxide, the heating is continued for 30 min., the flask is then cooled to room temperature and made up to the mark with water. The solution is filtered (Whatman no. 2 paper) and read in a colorimeter with an Ilford 624 (green) filter. The colour is stable for at least 3 weeks. When the method is used in enzyme assays, the reaction may be stopped by the addition of perchloric acid and a sample taken for analysis. In order to avoid undue dilution, a volume of 10 N-NaOH equivalent to the quantity of perchloric acid present is added; the final adjustment to a blue colour is made with 0·5 N-NaOH as above.

'*Micro*' *method*. The sample, placed in a test tube graduated at 5 ml., should contain approx. 20 μmoles of dipeptide or hydrolysis products. Ethanolic thymolphthalein (0·1%; 1 drop) is added and the solution made blue by the addition of 0·1 N-NaOH. Cobaltous phosphate suspension (2 ml.) is added. The remaining procedure is as described for the 'macro' method, with three successive additions of 0·25 ml. of 1 volume hydrogen peroxide. After making up to the mark it is preferable to centrifuge rather than filter the solution.

RESULTS

Factors influencing colour production

The addition of hydrogen peroxide in stages rather than in a single batch has the advantage that colour production is greater, more reproducible and linear with quantity of dipeptide. No advantage is gained from adding the peroxide in a greater number of increments than three, since reproducibility within the limits of use of the photo-electric colorimeter can be obtained by this technique. It is important to add the peroxide slowly, otherwise low results of poor reproducibility are obtained.

A systematic investigation of the effect of temperature on the colour-forming reaction was not attempted; however, unless the colour formation is carried out in a boiling-water bath, the solutions obtained are difficult to filter or to centrifuge. Furthermore, lowering the temperature causes increased colour production from glycine; this is disadvantageous since it reduces the precision of the method.

The effect of varying the time of incubation after the final addition of peroxide was investigated; increasing this reduces the colour production from solutions containing large amounts of glycine. Since no further reduction was found after 30 min., this was chosen as the most suitable time.

The effect of a number of added substances was also investigated. Some of the results obtained are shown in Table 1. It can be seen that the method is suitable for use in enzyme experiments in barbiturate buffers and for assaying preparations of sarcosomes and mitochondria, since added sucrose has no effect. A slight depression of colour formation is observed by the addition of 200 μmoles of phosphate. The readings can be corrected by simple proportion to bring them back to a calibration curve constructed in the absence of phosphate. There was no detectable depression of colour formation on the addition of 100 μmoles of phosphates.

Chromogenic compound. The absorption spectra of solutions obtained by carrying out the 'macro' method on 80 μmoles of glycylglycine and on 160 μmoles of glycine are shown in Fig. 1. The dipeptide complex has an absorption peak at 520 mμ with a smaller peak at 390 mμ. Alanylglycine, glycylalanine, alanylalanine and leucylglycine give

Table 1. *Colorimeter readings on mixtures of glycylglycine and glycine which simulate different degrees of hydrolysis of* 80 μ*moles of glycylglycine*

Addition	Colorimeter reading		
	0% hydrolysis	50% hydrolysis	100% hydrolysis
None	50·0	21·3	1·8
$CoCl_2$ (10 μmoles)	49·4	21·6	1·9
Na_2HPO_4 (200 μmoles)	47·8	20·5	1·9
Na_2HPO_4 (200 μmoles) corr.*	50·0	21·4	1·9
2% Sodium barbiturate (1 ml.)	49·5	21·6	—
1:5 Rat-muscle homogenate (6 mg./ml.; 1 ml.) + 20% perchloric acid (2·5 ml.)	50·2	—	1·8
20% (w/v) Sucrose (1 ml.)	49·2	21·4	1·3

* Multiplied by 50·0/47·8, the ratio of the colorimeter readings before hydrolysis in the absence and presence of phosphate.

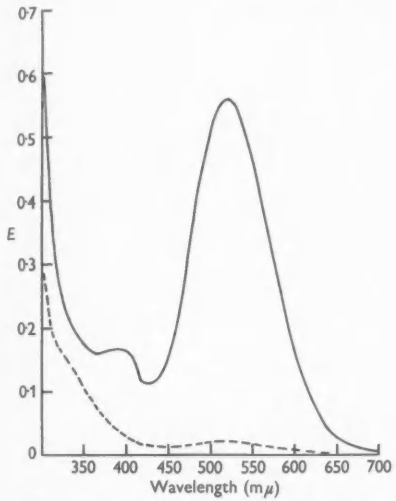

Fig. 1. Absorption spectra of oxygenated cobalt complexes of: ——, 80 μmoles of glycylglycine; – – –, 160 μmoles of glycine.

absorption spectra similar to glycylglycine. Methylation of the amino group, as in sarcosylglycine, reduced colour production and shifts the wavelength of maximum absorption to 530 mμ. Very little colour is given by glycinamide. Of the remaining dipeptides tested, all except those containing tyrosine residues have absorption maxima at 520 mμ. The colour production from a number of dipeptides is illustrated in Table 2. The optical density at 520 mμ does not always parallel the colorimeter reading with an Ilford 624 filter (maximum transmittance at 520 mμ); this is illustrated by the figures quoted for alanylglycine and leucylglycine in Table 2. One reason for this is that the absorption spectra produced from different dipeptides differ in the breadths of their absorption peaks; since the 624 filter has a rather broad transmittance curve, the colorimeter reading represents the integrated absorption over quite a wide spectral range and thus reflects the shape of the absorption peaks as well as their height.

The colour production of a number of amino acids and related compounds is illustrated in Table 3; it can be seen that the colour production from most of these substances is negligible. However, atypical colours are produced from some substituted amino acids and peptides; these are listed in Table 4. The absorption spectra produced from some peptides giving atypical results are shown in Fig. 2; DL-leucylglycine is included for the purpose of comparison.

Table 2. *Colour production obtained from 80 μmoles of some simple dipeptides*

Substance	Colorimeter reading (624 filter)	Optical density (520 mμ)
Glycylglycine*	50·3	0·560
DL-Alanylglycine*	51·8	0·584
DL-Leucylglycine*	52·9	0·574
Glycyl-DL-leucine*	32·5	—
Glycyl-DL-alanine*	50·4	0·560
DL-Alanyl-DL-alanine*	47·8	0·532
Glycyl-L-tryptophan	34·0	0·389
Glycyl-DL-phenylalanine*	36·5	0·411
Sarcosylglycine	38·3	0·408†
N-Ethylglycylglycine	19·4	—
Glycylproline	0·0	—

* These compounds give essentially the same absorption spectrum as glycylglycine.

† 0·420 at wavelength of maximum absorption (530 mμ).

Table 3. *Colour production from 80 μmoles of some representative amino acids and related substances, with an Ilford 624 filter*

Substance	Colorimeter reading
Glycine	2·7
Alanine	5·6
Valine	4·8
Leucine	2·7
Serine	3·2
Proline	2·5
Phenylalanine	1·2
Arginine	3·4
Aspartic acid	4·9
Glutamic acid	3·8
Asparagine	6·0
Glycinamide	9·3
Acetylglycine	0·2
Ammonium chloride	0·0

Linearity of colour production

For dipeptides alone, the graphs of colorimeter readings plotted against quantities of dipeptides are linear, either the 'macro' or 'micro' techniques (e.g. Fig. 3 being used). Deviations from the straight line give a measure of the precision of the method: in no case are deviations greater than 0·5 of a colorimeter unit obtained. This linearity is destroyed by the addition of amino acids, and hence, in any application of the method for observing the hydrolysis of dipeptides, calibration curves must first be constructed with known mixtures of the dipeptide and its hydrolysis products. Some examples of such curves are shown in Fig. 4; these curves are quite reproducible.

In order to investigate this non-linearity of the calibration curves, the effect of glycine on the colour production from a constant amount of glycylglycine was determined (Fig. 5). It can be seen that glycine reduces the amount of colour produced and that this reduction is very nearly

Table 4. *Colour production from 80 μmoles of some substances giving atypical results*

Substance	Colour produced	Colorimeter reading (624 filter)	Optical density (520 mμ)	Wavelength of max. absorption (mμ)	Optical density at max. absorption
Diglycylglycine	Orange	29·0	0·383	487	0·532
Glycyl-L-tyrosine	Orange–yellow	27·7	0·322	505	0·331
Sarcosylglycine	Bluer than glycylglycine	38·3	0·408	530	0·420
L-Tyrosine	Orange–yellow	30·3	—	—	—
L-Histidine	Orange–yellow	14·8	—	—	—
L-Tryptophan	Yellow	15·1	—	—	—
L-Cysteine	Yellow–green	42·8	—	—	—

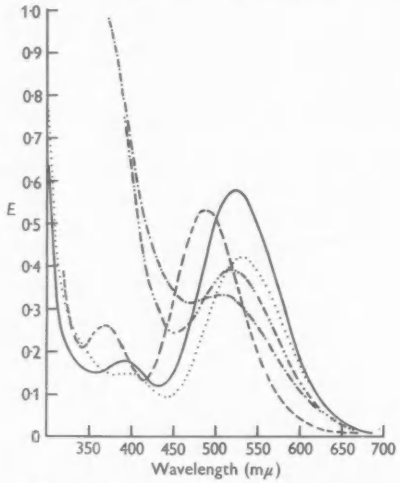

Fig. 2. Absorption spectra of oxygenated cobalt complexes of 180 μmoles of: ——, DL-leucylglycine; - - - - -, diglycylglycine; · · · ·, sarcosylglycine; —·—·—, glycyl-L-tyrosine; —· · —, glycyl-L-tryptophan.

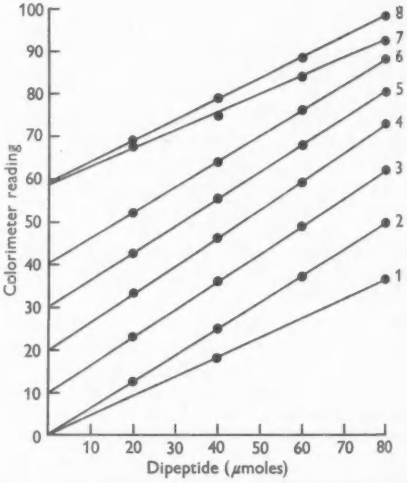

Fig. 3. Linearity of colour production with dipeptides. Graphs have been displaced vertically by the number of colorimeter units indicated in parentheses: 1, glycyl-DL-alanine (0); 2, glycylglycine (0); 3, DL-alanylglycine (10); 4, DL-leucylglycine (20); 5, glycyl-DL-alanine (30); 6, DL-alanyl-DL-alanine (40); 7, glycyl-DL-leucine (60); 8, sarcosylglycine (60).

proportional to the concentration of glycine present. Since the quantity of glycine present varies continuously along the calibration curve, the immediate cause of non-linearity in the calibration curves is apparent. The reduction in colour produced by added glycine might be due to the inhibition of the colour-forming reaction or to the formation of mixed complexes of amino acids and dipeptides. Spectrophotometric results have been obtained which suggest that the latter explanation is the correct one (Fig. 6). In the region 280–460 mμ the experimental solution has a greater extinction, and from 460 to 700 mμ a smaller extinction than is predicted by assuming additivity of extinction of the individual components. The simplest explanation of these results is that mixed complexes of amino acids and dipeptides are formed, and that these have a higher absorbency at wavelengths less than 460 mμ and a smaller extinction at wavelengths greater than 460 mμ than the sum of the corresponding quantities of simple complexes. Similar results have been obtained with alanylglycine–alanine and glycyl-alanine–glycine–alanine mixtures.

Further evidence of a qualitative nature has been obtained for the formation of mixed complexes. The addition of the cobaltous phosphate suspension to a mixture of a dipeptide and an amino acid causes the production of an olive-brown colour; this is not observed for either dipeptides or amino acids alone and it might well be associated with the formation of mixed complexes. This colour production is labile and disappears on heating in a boiling-water bath.

A disadvantage of non-linear calibration curves arises when the calibration curve based on one initial quantity of dipeptide has been determined, but is required for another initial quantity. However, since the non-linearity is probably due to the formation of mixed complexes, then provided that the ratio [amino acid]:[dipeptide] is held constant, the colorimeter reading should be proportional to [dipeptide], since the relative amounts of the complexes should be independent of the absolute concentration of dipeptide. Thus a graph of colorimeter reading against quantity of dipeptide is linear in the presence of amino acids provided that the ratio [dipeptide]:[amino acid] is held constant. For two calibration curves, each based on a

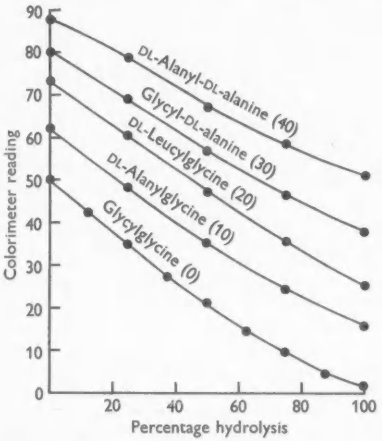

Fig. 4. Calibration curves for the determination of hydrolysis based on an initial 80 μmoles of dipeptides. Graphs are displaced vertically by the number of colorimeter units shown in parentheses.

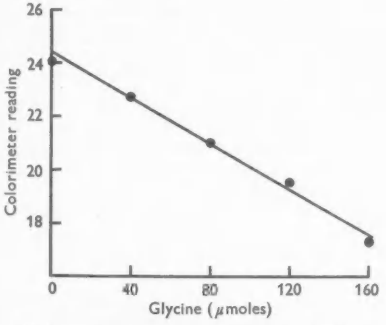

Fig. 5. Effect of glycine on colour production from 40 μmoles of glycylglycine.

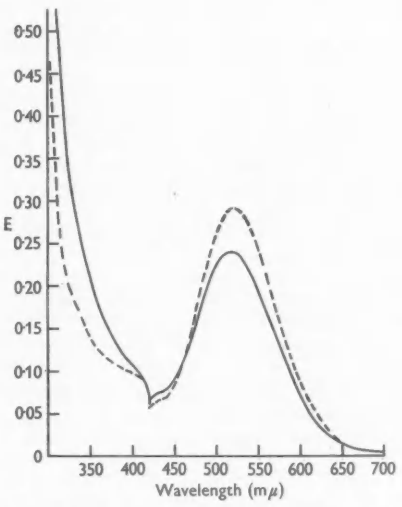

Fig. 6. Absorption spectrum obtained from 40 μmoles of glycylglycine + 80 μmoles of glycine: ——, experimental; - - - -, theoretical obtained by adding together the individual extinctions of the components.

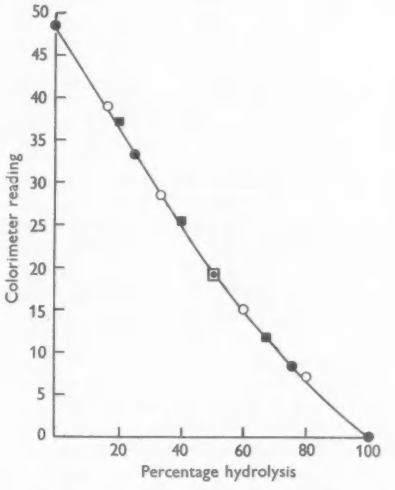

Fig. 7. Inter-relation of calibration curves based on different initial quantities of glycylglycine. Initial quantity of glycylglycine: ●, 80 μmoles; ○, 60 μmoles; ■, 50 μmoles. Experimental points based on the initial 60 and 50 μmoles were converted into the curve for 80 μmoles by simple proportion.

different initial quantity of dipeptide, the ratio [dipeptide]:[amino acid] at any given percentage hydrolysis is constant and independent of the initial quantity of dipeptide. Since the colorimeter reading is linear with quantity of dipeptide in the presence of amino acids when the ratio [dipeptide]:[amino acid] is constant, it follows that all the calibration curves based on different initial quantities of dipeptide should be interconvertible by simple proportion. To do this it is essential to use the 100% hydrolysis solution as a blank since amino acids alone give a non-stoicheiometric colour production. An example of such interconversion is shown in Fig. 7, where colorimeter readings of artificially prepared hydrolysis mixtures based on an initial 50 and 60 μmoles of glycylglycine have been converted so as to fall on a calibration curve based on an initial 80 μmoles of glycylglycine.

DISCUSSION

The success of the present method results from the use of cobaltous phosphate suspensions. No stoicheiometric relation between colour and amount of dipeptide was obtained with suspensions of cobaltous hydroxide in various buffers, including borate, or in the presence of glycerol; various oxidizing reagents other than hydrogen peroxide were also investigated. The chief merit of phosphate appears to lie in its ability to retard the oxidation of Co^{2+} ions and thus to allow full opportunity for oxygenation of the cobaltous complexes.

The method is rapid and accurate. Thirty or more estimations can be performed in one batch in 2–3 hr. Its reproducibility is such that individual determinations in the one colorimeter, but with different batches of reagents, have a standard deviation of 1·5–2% of the colorimeter reading. With care the percentage hydrolysis of dipeptides can be determined within the limits of precision of the colorimeter. Where precision of a lower order suffices, simplifications can be made; the time of final incubation can be cut and the hydrogen peroxide can be added in a single batch. Thus the method has obvious advantages over other methods which are available for observing hydrolyses, although its application is restricted, in the main, to dipeptides of simple, non-substituted amino acids.

Most amino acids give colours initially, but these are not stable during the subsequent heating. Very little colour is obtained from glycinamide, although this substance forms complexes with Co^{2+} ion of stability comparable with those of sarcosylglycine (Datta & Rabin, 1956). It is probable therefore that a free carboxyl group is essential for colour formation. No colour is obtained from glycylproline, although this substance forms complexes with Co^{2+} ion of stability greater than those of glycylglycine (Datta & Rabin, 1956). A possible explanation of this is that, in the formation of a coloured complex, ionization of the peptide hydrogen occurs as with the copper complexes of these compounds (Datta & Rabin, 1956).

SUMMARY

1. A colorimetric procedure for the determination of dipeptides is described.

2. It depends upon the formation of oxygenated cobalt complexes of the dipeptides in the presence of alkaline cobaltous phosphate suspensions.

3. It is applicable to a large number of dipeptides and has advantages of rapidity and accuracy.

4. It can be used for following the hydrolysis by acids, bases or enzymes of dipeptides of monoaminomonocarboxylic acids, but not for those containing cystein, tyrosine, tryptophan or histidine, since these amino acids themselves give appreciable colours under the conditions of the assay. Most other amino acids and ammonium salts cause no appreciable interference.

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Further Evidence on the Protein Complexes of Some Hyaluronic Acids

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This work follows from that of Blumberg & Ogston (1957). They showed that papain is a specific agent for degrading the protein part of the hyaluronic acid complex isolated from ox synovial fluid by filtration (Ogston & Stanier, 1950), and that when this protein is degraded marked changes in the non-Newtonian viscosity occur. This is associated with a marked decrease in the molecular weight and the hydrodynamic specific volume as well as changes in other physical characteristics, which indicate that the material has been depolymerized.

The correlation between the degradation of protein which forms the complex with hyaluronic acid and the change of non-Newtonian viscosity makes possible the use of papain as a reagent for detecting whether hyaluronic acid from any source contains protein in such a state as to determine its physicochemical properties. This test can be applied in the presence of free protein since free protein, its hydrolysis products, or excess of enzyme do not contribute significantly to the non-Newtonian viscosity of solutions which contain hyaluronic acid, and preliminary separation of the hyaluronic acid from other materials is therefore unnecessary. The test has been applied to several preparations containing hyaluronic acid from different sources.

A preparation of plasmin has been found to have effects on the viscosity of solutions containing the hyaluronic acid–protein complex similar to those produced by papain. This gives weight to the suggestion that changes in the protein part of the complex may have physiological and pathological significance.

A method based upon that of Scott (1955, 1956), for isolating acid polysaccharides by precipitating them with cetylpyridinium chloride, has been applied to synovial fluid. Preliminary results show that the product contains protein and appears to resemble closely the material isolated by filtration by the method of Ogston & Stanier (1950).

EXPERIMENTAL

Materials

Ox synovial fluid. This was collected from the slaughter-house of the Oxford and District Co-operative Society Ltd., as described by Ogston & Stanier (1950).

* Fellow of the Arthritis and Rheumatism Foundation.

Hyaluronic acid complex by the filtration process. This was prepared by the method of Ogston & Stanier (1950). The complex was washed 16 times with buffer on a sintered-glass filter of 1 μ average pore diameter. The resulting solution showed only a single, very sharp boundary in the ultracentrifuge, and no sign of free protein.

Ox serum albumin. This was prepared as described by Blumberg & Ogston (1956).

Human synovial fluids. These were supplied by Mr R. G. Taylor of the Wingfield–Morris Orthopaedic Hospital, Oxford. They were taken at operation from patients with traumatic effusions of the knee joint and were preserved under sterile conditions and stored at 4°. There was no evidence of chronic arthritic disease in any of the patients; all the synovial fluids gave a normal type of mucin clot on acidification with 10% (w/v) acetic acid.

Extract of human umbilical cord. Umbilical cords were collected from the Radcliffe Infirmary, Oxford, within a few hours of delivery. Each cord was quickly washed free of blood and the blood vessels were removed by dissection. Hyaluronic acid was extracted from the remaining tissue by the method of Fessler (1956); the tissue was coarsely chopped and placed overnight in a closed jar with sufficient buffer (pH 7, 0·067 M-phosphate) just to cover it, the entire contents of the jar were then centrifuged in a Spinco Model L ultracentrifuge for 2 hr. at 73 000 *g*. The supernatant contained hyaluronic acid and free protein. Fessler (1956) showed that material prepared in this way contains no detectable collagen fibres. Precipitation with cetylpyridinium chloride (CPC) by the method of Scott (1955, 1956) showed that it contained no more than a trace of chondroitin sulphate. Fessler (1956) showed that re-extraction of the tissue with buffer would yield further amounts of polymeric material, but only the first extract was used in this work.

Extract of ox vitreous humour. Vitreous humour was obtained from cattle eyes given to us by Dr Antoinette Pirie of the Nuffield Laboratory of Ophthalmology, Oxford. Within a few hours of death each eye was injected with 0·2 ml. of a 0·5 M-solution of ethylenediaminetetra-acetic acid (EDTA) neutralized to pH 7 in order to prevent oxidative depolymerization of the hyaluronic acid by the copper ion–ascorbic acid system (Bárány & Woodin, 1955). The eye was freed from the muscle attachments and an incision made along the equator of the sphere. The vitreous humour was slipped out, and freed from the lens and iris. The whole vitreous was then centrifuged for 2 hr. at 73 000 *g* in the Spinco Model L ultracentrifuge at 5°. Under these conditions any fibrous material is sedimented and the supernatant solution contains hyaluronic acid in low concentration (Woodin & Boruchoff, 1955).

Streptococcal hyaluronic acid. A preparation of streptococcal hyaluronic acid, stated to be free from protein, was given to us by Dr S. P. Halbert, of Columbia University, New York. The method of preparation has been briefly

stated by Halbert, Swick & Sonn (1955). A solution of this material showed no absorption maximum in the region of 280 mμ, which confirms the absence of protein. Stock solutions of 0·75% were made in the appropriate buffer.

Hyaluronic acid prepared from synovial fluid with cetylpyridinium chloride. This was done by a modification of the method of Scott (1955, 1956). Drs D. Jackson and J. E. Scott kindly helped us in the development of these modifications. Ox synovial fluid (100 ml.) was dialysed against distilled water for 24–48 hr. with frequent changes of the diffusate. Cetylpyridinium chloride (0·5% in water) was added slowly from a burette until the formation of a clot was complete and the solution appeared to lose its elastoviscosity. (This was detected by swirling the solution and observing if there was any 'bounce-back' when swirling was stopped.) The supernatant solution was drained off and the clot washed with fifteen changes of 50 ml. each of water. It was then redissolved in a solution composed of methanol, saturated sodium chloride and water (10:33:57, by vol.). Re-solution was helped by warming to 37°. Because of the strong antibacterial action of CPC there is little danger of bacterial contamination. The excess of CPC was then removed by adding small quantities of washed fuller's earth until foaming on agitation ceased. The fuller's earth was removed by centrifuging at 2000 rev./min. for 2 hr. The material was dialysed against the appropriate buffer for 2 days and centrifuged at 73 000 *g* for 45 min. to remove all suspended particles.

Papain. This was twice crystallized, from crude papain B.P.C. (British Drug Houses Ltd.), by the method of Kimmel & Smith (1954). The product was freeze-dried and stock solutions were made by dissolving the dry material in buffer to 1 g./100 ml.

Plasmin. A sample of plasmin prepared by a modification (Biggs & Macfarlane, 1953) of the method of Rocha e Silva & Rimington (1948) was kindly presented to us by Dr E. Bidwell of the Department of Pathology, The Radcliffe Infirmary, Oxford. The activity of the enzyme was determined by the clot-lysis method of Rocha e Silva & Rimington (1948); the value obtained was 3·6 mg. by their definition.

Hyaluronidase. Benger hyalase (Benger Laboratories, Holmes Chapel, Cheshire) with an activity of 1500 international units/mg. was used.

Buffers. For the experiments with papain and plasmin and for the extraction of umbilical cord the buffer was 0·0453 M-Na_2HPO_4, 0·0213 M-KH_2PO_4 (pH 7); for preparative work and for the physical measurements the buffer was 0·0077 M-Na_2HPO_4, 0·0023 M-KH_2PO_4, 0·2 M-NaCl (pH 7·3).

Methods

Viscosity. The non-Newtonian viscosity was measured with the Couette viscometer of Ogston & Stanier (1953*a*) at 25° with velocity gradients between 0·25 and 40 sec.$^{-1}$. Viscosity at high-velocity gradient was measured with an Ostwald viscometer of 2 ml. capacity and water-time of 11 sec. Intrinsic viscosity measurements were made in an Ubbelohde (1936) viscometer with a capacity of 20 ml. and a water-time of 110 sec.

Sedimentation. This was measured in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. by the method of Cecil & Ogston (1948). The optical system was Bäldwin's (1953) modification of the diagonal-schlieren method of Philpot (1938).

Treatment of preparations of the hyaluronic acid complex with papain. Papain (1 ml. of 1% solution) was added to 10 ml. of solution of the substrate in buffer, pH 7. The buffer contained KCN and EDTA to activate the papain; the final concentration of each in the digestion mixture was 5 mM. Incubation was at 4° for the following times: whole ox synovial fluid, 24 hr.; ox synovial fluid enriched with protein, 30 hr.; human synovial fluid, 24 and 48 hr.; umbilical-cord extract, 24 hr. (followed by 30 min. at 25°). Each mixture was brought to 25° for 1 hr. before measurement in the Couette viscometer. Control experiments were done with the papain omitted.

Treatment of hyaluronic acid complex and ox synovial fluid with plasmin. Plasmin was dissolved in water and mixed with substrate dissolved in the buffer, pH 7. The conditions were: for hyaluronic acid complex, 100 mg. of plasmin in 4 ml. was added to 10 ml. of substrate and incubated at 25° for 6 hr.; for synovial fluid, 26 mg. of plasmin dissolved in 2·5 ml. was added to 10 ml. of substrate and incubated at 25° for 20 hr. Control experiments were done with plasmin omitted.

Mucin. The concentration of hyaluronic acid complex in ox synovial fluid was determined from the dry weight of the mucin clot formed upon acidification (Ogston & Stanier, 1952). Attempts were made to use the same method, after addition of serum albumin, to estimate the concentration of hyaluronic acid prepared by precipitation with CPC; however, with this material, the results disagreed with those obtained by other methods, and the method was abandoned.

The formation of mucin was also used to judge qualitatively the state of the hyaluronic acid in various preparations, as described by Ropes & Bauer (1953). Ox serum albumin was added to those preparations which did not contain sufficient free protein for mucin formation. The results were graded according to the standard practice (Ropes & Bauer, 1953): group I, firm, ropy clot, with complete clearing of the supernatant solution; group II, clot formed, but not as firm as in group I, wispy edges to the clot, incomplete clearing of the supernatant; group III, no clot, but a dispersed white precipitate.

It was found possible to grade similarly the clots formed by adding 0·5% CPC in water to 2 ml. of the sample mixed with 2 ml. of water, until clotting was complete.

Estimation of dry weight. Solutions were dialysed against fifteen changes of glass-distilled water for 7 days. Weighed samples of the solution were then dried to constant weight at 105°.

Total nitrogen. This was measured by the micro-Kjeldahl method. A selenium catalyst (Fawcett, 1954) with 6 hr. digestion and a Markham (1942) still were used.

RESULTS

Effects of papain on viscosity

Ox synovial fluid. Blumberg & Ogston's (1957) results were obtained with hyaluronic acid complex isolated from ox synovial fluid by filtration. When whole ox synovial fluid was treated with papain, the non-Newtonian viscosity was similarly lowered, though rather more slowly than with the isolated complex; this was to be expected, since the free protein of the synovial fluid would compete with

the protein of the complex for the papain. Next, ox serum was mixed with ox synovial fluid to give a final concentration of 4 g. of protein/100 ml., which is about the same as that of protein in human synovial fluid from acutely traumatized joints (Johnston, 1955*a*); here too the papain lowered the non-Newtonian viscosity, though more slowly still. Apart from the effect of excess of protein on the rate of change of viscosity with time, the results obtained were similar to those described by Blumberg & Ogston (1957).

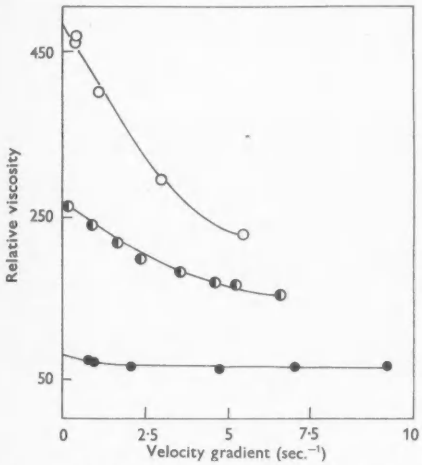

Fig. 1. Effect of papain on acute traumatic human synovial fluid. Variation of relative viscosity with velocity gradient was measured in the Couette viscometer at 25°. ○, Synovial fluid control; ◐, synovial fluid treated with papain for 24 hr. at 4°; ●, synovial fluid treated with papain for 48 hr. at 4°.

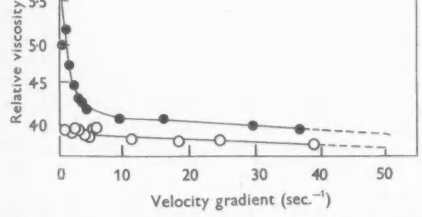

Fig. 2. Effect of papain on hyaluronic acid complex extracted from human umbilical cord. Variation of relative viscosity with velocity gradient was measured in the Couette viscometer at 25°. ●, Untreated control; ○, treated with papain for 24 hr. at 4° and 30 min. at 25°.

Human synovial fluid. Two different samples of human synovial fluid were treated with papain; in each case the non-Newtonian viscosity was considerably reduced (Fig. 1).

Hyaluronic acid from umbilical cord. In two different preparations similar reduction of non-Newtonian viscosity was found (Fig. 2).

Hyaluronic acid from vitreous humour. The preparations showed no non-Newtonian viscosity, at the concentration used (cf. Woodin & Boruchoff, 1955); the effect of papain was therefore not tested.

Streptococcal hyaluronic acid. This material, in concentrations about five times that of a synovial fluid, showed no non-Newtonian viscosity. Since it was known to be protein-free, the effect of papain on the viscosity (measured in the Ostwald viscometer) was tested in order further to exclude the possibility that the action of papain might be on the hyaluronic acid itself rather than on the protein (Blumberg & Ogston, 1957). No effect of papain on the viscosity could be observed. When a 0·75% solution was treated with 1 mg. of hyalase at 25° the viscosity (measured in the Ostwald viscometer) fell from 12·7 to 1·7 in ½ hr.

Hyaluronic acid complex isolated from ox synovial fluid with cetylpyridinium chloride. This material showed marked non-Newtonian viscosity (see below). Incubation with papain resulted in a loss of non-Newtonian viscosity similar to that observed with hyaluronic acid complex made by filtration (Blumberg & Ogston, 1957) and with the preparations described above.

Mucin and cetylpyridinium chloride clots

Group I (normal) mucin clots were obtained with hyaluronic acid complex prepared by filtration or by precipitation with CPC, with whole ox synovial fluid, with human synovial fluid from traumatized joints and with the hyaluronic acid extracted from umbilical cord. After treatment of any of these with papain, the clot obtained was group II or III. Group II or III clots were obtained also after treating hyaluronic acid complex or whole synovial fluid with plasmin. Synovial fluids from cases of rheumatoid arthritis, the protein-free streptococcal hyaluronic acid and the hyaluronic acid of vitreous humour, all gave group II or III clots.

The characters of the clots obtained with CPC were in all cases closely similar to those of the mucin clots.

Effect of plasmin on hyaluronic acid and on whole ox synovial fluid

The effect of plasmin on the non-Newtonian viscosity were similar to those produced by papain (Fig. 3). Simultaneous proteolysis of the protein of the hyaluronic acid complex was demonstrated by the method of Anson (1938).

Properties of hyaluronic acid prepared from ox synovial fluid by use of cetylpyridinium chloride

The ultraviolet absorption of the first samples prepared by this method showed that they contained protein in amount comparable with that present in samples prepared by filtration. Also, they showed marked non-Newtonian viscosity, which was reduced by treatment with papain. In view of these surprising results, and of the possible identity of material made in this way with the complex obtained by filtration, its properties were more thoroughly investigated.

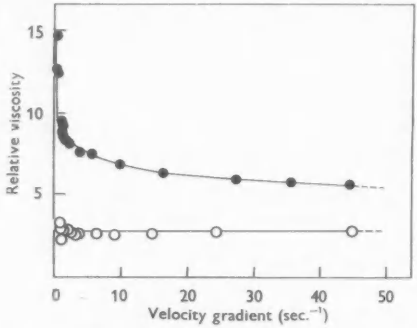

Fig. 3. Effect of plasmin on whole ox synovial fluid. Variation of relative viscosity with velocity gradient was measured in the Couette viscometer at 25°. ●, Untreated synovial fluid control; ○, synovial fluid treated with plasmin (0·4 %) for 10 hr. at 25°.

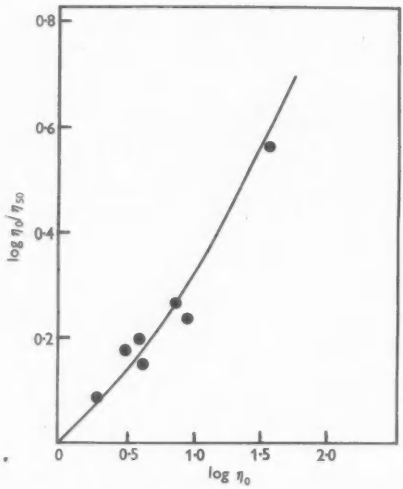

Fig. 4. Curve of log η_0/η_{50} against log η_0 for undegraded ox synovial fluid (taken from Fessler *et al.* 1954). ●, Values for hyaluronic acid complex prepared from ox synovial fluid by the CPC method.

Non-Newtonian viscosity. The variation of relative viscosity with velocity gradient was measured in a number of samples. The degree of non-Newtonian viscosity was measured by the relationship of the ratio η_0/η_{50} (relative viscosities extrapolated respectively to velocity gradients zero and 50 sec.$^{-1}$) to η_0 (Fessler, Ogston & Stanier, 1954). The log values for these ratios are shown, in Fig. 4, plotted against log η_0; the full line represents the results obtained by Fessler *et al.* for whole ox synovial fluid. It appears that the CPC product has normal non-Newtonian viscosity. Three of the samples studied contained no detectable amount of CPC.

Protein content. This was measured by comparing the extinction at 280 mμ with the dry weight of the sample. The specific extinction coefficient at 280 mμ of the protein in the complex was taken as 14, based upon measurements made on hyaluronic acid complex made by filtration. The proportions of protein in three samples were 29·8, 23·3 and 23·5 %. Ogston & Stanier (1952) found 25·7 % for hyaluronic acid complex made by filtration. The proportions of CPC in the products were judged approximately by the extinction at 257 mμ ($E^{1\%}_{1cm.}$ for CPC = 111 at 257 mμ); values varied between 1 % and zero.

Sedimentation and intrinsic viscosity. The CPC product gave a boundary diagram (Fig. 5) indistinguishable from that given by hyaluronic acid complex; there was no sign of any free protein. The sedimentation of a sample of CPC product was compared with that of hyaluronic acid complex in the same synovial fluid from which it was prepared. Sedimentation coefficients were measured at several concentrations in each case and extrapolated to zero concentration by plotting $1/S$ against concentration. The same plots gave values of d $(1/S)$/dc (Table 1). The latter are, however, not directly comparable because of the presence of free protein in the synovial fluid, which affects the rate of sedimentation of the hyaluronic acid. Correction for this (Johnston, 1955*b*; Fessler *et al.* 1954) gives the corrected value shown in Table 1. The intrinsic viscosities of the two materials were compared, by use of the Ubbelohde viscometer; the results are given in Table 1.

Calculation of values of d $(1/S)$/dc and the intrinsic viscosities requires knowledge of the concentrations of the hyaluronic acid complex. For the synovial fluid, this was determined by dry weight of mucin (Ogston & Stanier, 1952); for the CPC product by determining the dry weight directly.

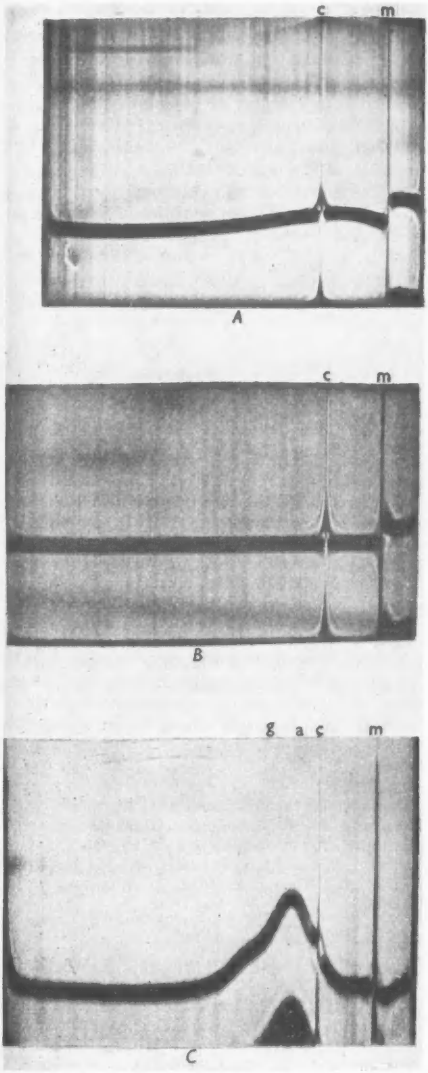

Fig. 5. Schlieren diagram of sedimenting ox synovial fluid and hyaluronic acid complex isolated from it by the CPC and filtration methods. Measurements were made at 900 rev./sec. at 26° (approx.) in the pH 7·3 buffer. The photographs were taken 30 min. after reaching speed. Sedimentation occurs from right to left. *A*, Hyaluronic acid complex prepared by the CPC method; *B*, hyaluronic acid complex prepared by filtration; *C*, whole ox synovial fluid. From right to left the boundaries are: *m*, meniscus; *c*, hyaluronic acid complex; *a*, albumin; *g*, globulin.

DISCUSSION

Complexes of hyaluronic acids with protein. The lack of action of papain on the protein-free bacterial hyaluronic acid confirms Blumberg & Ogston's (1957) conclusion that the effect of papain is exclusively on protein associated with the hyaluronic acid, and any effect of papain on the properties of hyaluronic acid must therefore be ascribed to proteolysis. In view of this conclusion, the effects of papain on the non-Newtonian viscosity and on the nature of the mucin and CPC precipitates of hyaluronic acids present in human synovial fluid and in umbilical cord provide strong evidence that they, like the hyaluronic acid of ox synovial fluid, exist as complexes with protein. The resemblance of the hyaluronic acid from vitreous humour to the protein-free bacterial hyaluronic acid, in its failure to show non-Newtonian viscosity and in giving group II or III mucin and CPC clots, suggests that it is not in the form of a complex with protein. This is consistent also with the experiments of Woodin & Boruchoff (1955).

Action of plasmin. Blumberg & Ogston (1957) suggested that proteolysis should be considered as a possible cause of pathological changes in tissues which contain hyaluronic acid. This suggestion is strengthened by the finding that a proteinase derived from human tissues can attack the protein which is present in the hyaluronic acid complex. The changes of non-Newtonian viscosity brought about by proteolysis could affect the efficiency of lubrication in joints (Ogston & Stanier, 1953*b*, *c*; Barnett, 1956); reduction of non-Newtonian viscosity of synovial fluid in rheumatoid arthritis has been reported by Sundblad (1954), Fessler *et al.* (1954), Johnston (1955*c*) and Levine & Kling (1956).

Mucin and cetylpyridinium chloride clots. Since the characters of mucin and CPC clots were in all cases closely similar, it is possible that clot formation with CPC might be used as an alternative, more convenient, test. [Di Ferrante (1956) has described a turbidimetric measurement of acid

Table 1. *Comparison of sedimentation and viscosity measurements on whole ox synovial fluid and on hyaluronic acid prepared from it by precipitation with cetylpyridinium chloride*

The value of $d(1/S_{20,w})/dc$ for synovial fluid has been corrected for the effect of free protein (see text).

	Intrinsic viscosity (g./100 ml.)$^{-1}$	$S^{\circ}_{20,w}$ (s)	Variation of $S_{20,w}$ with concentration $d(1/S_{20,w})/dc$ s^{-1} (g./100 ml.)$^{-1}$
Synovial fluid	25·0	10	2·4
CPC preparation	23·6	10	2·3

mucopolysaccharides with a quaternary ammonium compound.] The character of the mucin clot has been thought to be an index of the degree of polymerization of hyaluronic acid. Our experiments suggest that the presence and integrity of protein, in the form of a complex with the hyaluronic acid, may play an important part in determining the character of the clot. The fact that synovial fluid of rheumatoid arthritis gives group II or III clots, resembling those given by protein-free hyaluronic acids, or by protein-containing hyaluronic acids after proteolysis, supports the possibility that the protein part of the complex may be affected in this condition.

Separation of hyaluronic acid with cetylpyridinium chloride. The properties of hyaluronic acid prepared from ox synovial fluid appear closely similar to those of the native hyaluronic acid (and the hyaluronic acid complex prepared by the filtration process) in the following respects: non-Newtonian viscosity, intrinsic viscosity, sedimentation properties and content of protein. Further work, particularly analytical, is required to determine whether these products are in fact identical. If so, the preparation of hyaluronic acid complex will be greatly eased, particularly from the sources such as human synovial fluid where the particles are too small to be retained by the filters at present available.

The facts that a hyaluronic acid prepared by the CPC method retains the same amount of protein as does the material prepared by the completely independent filtration process (Ogston & Stanier, 1950) and that the physical integrity of the CPC-prepared material is dependent on the presence of this protein (i.e. its non-Newtonian viscosity is lost when the protein is degraded by papain digestion) lend additional strength to the argument that hyaluronic acid from ox synovial fluid occurs as a complex with protein. These experiments show how physical criteria can be used as a guide to the isolation of undegraded material from its natural source.

SUMMARY

1. Digestion with papain in conjunction with the measurement of non-Newtonian viscosity has been used as a test for the presence of protein in the form of a complex with hyaluronic acid derived from several sources. This test shows that hyaluronic acid exists as a complex with protein in human synovial fluid and human umbilical cord, as well as in ox synovial fluid. Hyaluronic acid from ox vitreous humour shows no non-Newtonian viscosity and this test therefore provides no evidence that it contains protein.

2. Papain has no detectable action on bacterial hyaluronic acid free from protein.

3. The character of the mucin clot is well correlated with the non-Newtonian viscosity; hyaluronic acid–protein complexes give normal (group I) clots, but abnormal (group II or III) clots after digestion with papain. Hyaluronic acid from vitreous humour gives an abnormal clot.

4. Plasmin has effects similar to those of papain, both on non-Newtonian viscosity and on the character of the mucin clot.

5. Precipitation of hyaluronic acid from ox synovial fluid with cetylpyridinium chloride gives a protein complex indistinguishable from that prepared by filtration and present in the native fluid.

6. The bearing of these results on the state of hyaluronic acid in pathological conditions is discussed.

We are grateful to Professor Eino Kulonen for advice and assistance during part of this work.

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Acidic Peptides of the Lens

3. THE STRUCTURE OF OPHTHALMIC ACID

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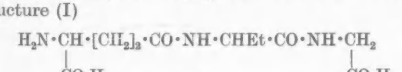
The investigation of the peptides in the lens has led to the discovery of a new tripeptide named ophthalmic acid. Earlier papers (Waley, 1956, 1957*b*) have described the isolation of this peptide, and provided some evidence for its structure. Acid hydrolysis gave glutamic acid, α-amino-*n*-butyric acid and glycine; glutamic acid provided the *N*-terminal amino acid residue, and glycine the *C*-terminal one. Assuming that the amino acids present in the hydrolysate are also those present in the tripeptide, these results establish the sequence of amino acid residues, and the only other question is whether it is the α- or the γ-carboxyl group of glutamic acid which is engaged in peptide-bond formation. Evidence on this point has been provided by measurements of mobilities in paper electrophoresis at pH 4. From the values given in Table 1 for several peptides of known structure it is seen that the γ-linked peptides of glutamic acid have mobilities over twice as large as the α-linked ones, and that ophthalmic acid has the mobility expected for a γ-linked peptide. This leads to the structure (I)



(I)

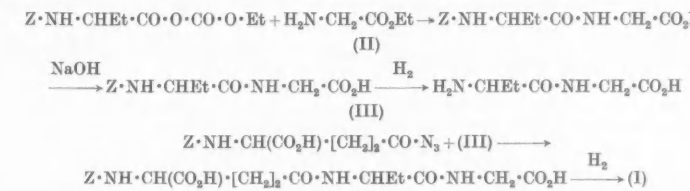
for ophthalmic acid. As there was insufficient material isolated from the lens for elementary analysis, the structure (I) is based on the assumptions that no undetected groups are present, that the amino acids liberated on hydrolysis have been correctly identified (by paper chromatography) and that hydrolysis has only liberated and not altered the amino acids forming the peptide. It was to test these assumptions that the synthesis of ophthalmic acid was undertaken; a preliminary account of this work has been given (Waley, 1957*a*). The preparation of γ-glutamyl-α-amino-*n*-butyric acid is also described below; this dipeptide was used in identifying the products from the action of carboxypeptidase on ophthalmic acid (Waley, 1957*b*).

In the synthesis of a γ-linked tripeptide of glutamic acid it is convenient to avoid protecting the α-carboxyl group, and this means that the glutamic acid residue must be incorporated last. So the synthetic route chosen was the one outlined in Scheme 1.

Table 1. *Electrophoretic mobility* (μ) *of peptides of glutamic acid*

Electrophoresis was carried out on the polythene-covered marble slab previously described (Waley, 1956) on Whatman no. 3MM paper at a potential gradient of 11·1 v/cm. for 6·25 hr. in the pyridine–acetic acid buffer, pH 4. The mobilities were corrected for electroendosmosis with *N*-(2:4-dinitrophenyl)ethanolamine as marker; the physicochemical interpretation of mobility measurement in paper electrophoresis has been discussed by Svensson (1956) and by Synge (1957).

Peptide	$10^5\mu$ (cm.^{2} v^{-1} sec.$^{-1}$)
α-L-Glutamyl-L-valine	2·8
α-L-Glutamyl-L-tyrosine	3·3
Glutamic acid	5·3
γ-L-Glutamyl-L-tyrosine	6·9
Ophthalmic acid	7·2
γ-L-Glutamyl-L-valine	7·5
Glutathione (disulphide form)	7·6
γ-L-Glutamylglycine	8·6



(II)

(III)

(IV)

(Z = $C_6H_5\cdot CH_2\cdot O\cdot CO$)

Scheme 1

Benzyloxycarbonyl-L-α-amino-*n*-butyrylglycine ethyl ester (II) was prepared in satisfactory yield by the mixed-anhydride method; although racemization may attend the use of mixed anhydrides from benzyloxycarbonyldipeptides, no case of racemization has been reported with the mixed anhydrides from benzyloxycarbonylamino acids (Waley & Watson, 1954; North & Young, 1955). The original description of the preparation of mixed anhydrides with ethyl hydrogen carbonate recommends allowing the reaction mixture to stand for about 10 min. at 0° to ensure formation of the anhydride (Boissonnas, 1951). Recently it has been suggested that this step should generally be omitted (Hooper, Rydon, Schofield & Heaton, 1956), as sometimes the mixed anhydrides are formed rapidly and rapidly decompose. But the protected dipeptide (II) was obtained in low yield, and much benzyloxycarbonyl-α-amino-*n*-butyric acid was recovered, when insufficient time was allowed for the formation of the mixed anhydride.

Saponification of (II), followed by hydrogenation, gave L-α-amino-*n*-butyrylglycine (III), which crystallized readily. Benzyloxycarbonyl-γ-L-glutamyl hydrazide was converted into the azide and condensed with the dipeptide (III) under conditions similar to those used in the synthesis of other γ-linked peptides of glutamic acid (Waley, 1955). The crude product, which was clearly impure, was probably contaminated with the isomeric α-glutamyl peptide; some isomerization of the γ-azide normally occurs (Sachs & Brand, 1954; Rowlands & Young, 1957). Fortunately, the isomers can usually be separated by fractional extraction from an organic solvent by aqueous alkali (LeQuesne & Young, 1950*a*), and the γ-linked compound was readily obtained pure by this method. The by-products, separated at this stage, were hydrogenated: the main constituent was glutamic acid, but there was also a compound with an electrophoretic mobility about half that of glutamic acid, and this suggests (see Table 1) that this compound is the α-linked isomer of (I). The main product was hydrogenated to give γ-L-glutamyl-L-α-amino-*n*-butyrylglycine (I); in the earlier experiments this was isolated as the copper salt, which crystallizes very readily, and reconverted into the tripeptide which could then be obtained crystalline; this was also the procedure used in the later stages of the isolation of ophthalmic acid from the lens (Waley, 1957*b*). With crystalline material to act as seeds, however, the copper-salt stage can be omitted.

The synthetic tripeptide was compared with, and found indistinguishable from, the ophthalmic acid from the lens, by the use of paper chromatography and paper electrophoresis. Final proof of identity was provided by comparison of the X-ray powder diagrams of the free tripeptides (for which I am indebted to Mrs D. M. Hodgkin, F.R.S., and Dr Beryl Oughton, whose report is given in the Experimental section). Ophthalmic acid from the lens has not been isolated in sufficient quantity to determine its optical rotation; it is assumed that the glutamic acid and the α-amino-*n*-butyric acid have the L-configuration.

The structure of ophthalmic acid (I) is clearly very similar to that of glutathione. Indeed, the only difference is the replacement of the thiol group in glutathione by a methyl group, and the thiol and methyl groups are nearly the same size. Thus whereas ophthalmic acid is devoid of the special reactivity of glutathione associated with the thiol group, the two tripeptides are probably very similar in shape. Their R_F values in paper chromatography, and mobilities in paper electrophoresis, are also very similar, and their specific rotations are not very different.

The biological role of ophthalmic acid may well be connected with its similarity to glutathione. It is well known that structurally similar compounds often have antagonistic biological properties (Woolley, 1952), so ophthalmic acid may behave as an anti-coenzyme in those reactions in which glutathione is a coenzyme.

EXPERIMENTAL

Melting points are uncorrected. The analyses and optical rotations were carried out by Weiler and Strauss, Oxford. Details of the paper chromatography are given in Table 2.

L-α-Amino-n-butyric acid. The preparation by the action of Raney nickel on L-methionine has been described (Fonken & Mozingo, 1947; Vogler, 1947; Berridge, Newton & Abraham, 1952). The use of freshly prepared active catalyst made by the method of Pavlic & Adkins (1946) enabled the reaction to be completed in 16 hr. at room temperature; the yield was 72%. Most of the L-α-amino-*n*-butyric acid used was bought from the California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles, California, U.S.A.

Benzyloxycarbonyl-L-α-amino-n-butyric acid. Benzyl chloroformate (10·2 ml.) and N-NaOH (10·5 ml.) were added dropwise with stirring and cooling to L-α-amino-*n*-butyric acid (4·3 g.) in 4N-NaOH (10·5 ml.). After 45 min., the mixture was extracted with ether, acidified to pH 2,

Table 2. *R_F values of peptides*

Compounds were chromatographed on Schleicher and Schüll no. 2034*b* paper (Carl Schleicher und Schüll, Dassel, Germany) with *n*-butanol–acetic acid–water (40:9:20, by vol.).

Substance	R_F
Alanine	0·35
α-Amino-*n*-butyric acid	0·44
α-Amino-*n*-butyrylglycine	0·35
γ-L-Glutamyl-α-amino-*n*-butyric acid	0·44
γ-L-Glutamyl-α-amino-*n*-butyrylglycine	0·32

and the product was isolated by extraction with ether. After evaporation of the ether, the residue was dried by addition of acetone–benzene (1:1) and evaporation of the solvent. The syrup was dissolved in dry ether, and light petroleum (b.p. 40–60°) was added; *N-benzyloxycarbonyl-L-α-amino-n-butyric acid* crystallized in acicular plates, m.p. 78–79°, $[\alpha]_D^{16}$ – 32° (*c*, 2·8 in ethanol); yield 5·3 g., 54%; the m.p. was not raised by recrystallization from ether–light petroleum (b.p. 40–60°) (Found: C, 60·6; H, 6·4; N, 5·9. $C_{12}H_{15}O_4N$ requires C, 60·7; H, 6·4; N, 5·9%).

Benzyloxycarbonyl-L-α-amino-n-butyrylglycine ethyl ester (II). Ethyl chloroformate (2·19 ml.) was added to a stirred solution of benzyloxycarbonyl-L-α-amino-*n*-butyric acid (5·46 g.) and tri-*n*-butylamine (distilled over NaOH; 5·48 ml.) in dry toluene (70 ml.) at −5°. After keeping the solution for 5 min. at this temperature (or for 14 min. at −15°) glycine ethyl ester, prepared from glycine ethyl ester hydrochloride (3·97 g.) and tri-*n*-butylamine (6·78 ml.) in dry $CHCl_3$ (82 ml.) was added, and the mixture kept for 1 hr. at −5°, and then for 3 hr. at room temperature. The solution was washed successively with water, 2% (w/v) $NaHCO_3$ and finally water, dried (Na_2SO_4) and concentrated to a volume of 60 ml.; *N-benzyloxycarbonyl-L-α-amino-n-butyrylglycine ethyl ester* separated, and was recrystallized from ethyl acetate–light petroleum (b.p. 60–80°); yield 4·5 g. (61%) of needles, m.p. 114°, $[\alpha]_D^{16}$ – 25° (*c*, 2·7 in ethanol) (Found: C, 60·1; H, 7·0; N, 8·7. $C_{16}H_{22}O_5N_2$ requires C, 59·7; H, 6·9; N, 8·7%).

L-α-Amino-n-butyrylglycine (III). 0·5N-Sodium hydroxide (45·4 ml.) was added to benzyloxycarbonyl-L-α-amino-*n*-butyrylglycine ethyl ester (6·65 g.) in acetone (66 ml.). After the solution had been kept for 2 hr. the acetone was removed at room temperature, and the alkaline solution extracted thrice with ethyl acetate. The ethyl acetate extracts were washed with water and the aqueous layer was added to the aqueous alkaline solution, which was then neutralized with N-HCl (22·7 ml.). The oil was isolated by extraction with ethyl acetate, the ethyl acetate solution freed from Cl^- ions by washing thrice with a little water, and the solvent distilled. The residue was dissolved in acetic acid (20 ml.) and water (10 ml.) and hydrogenated in the presence of palladium black (1 g.); after 4 hr. reduction was complete. The filtered solution was evaporated, the acetic acid removed by adding water and evaporating again, and the residue dried by the addition of acetone (10 ml.) and benzene (10 ml.) and distilling the solvents. Trituration under acetone gave the crystalline *dipeptide* (yield 2·5 g., 76%), m.p. 222° (decomp.), sufficiently pure for the next stage. The analytical specimen, recrystallized from aq. propan-2-ol, had m.p. 225° (decomp.), $[\alpha]_D^{16}$ +72° (*c*, 2·8 in water); (Found: C, 44·7; H, 7·7; N, 17·8. $C_8H_{12}O_3N_2$ requires C, 45·0; H, 7·5; N, 17·5%).

Benzyloxycarbonyl-γ-L-glutamyl-L-α-amino-n-butyrylglycine (IV). Sodium nitrite (1·09 g.) in water (10·9 ml.) was added during 7 min. to a cooled, stirred solution of benzyloxycarbonyl-γ-L-glutamyl hydrazide (LeQuesne & Young, 1950*b*) (3·21 g.) in water (27 ml.) containing 6N-HCl (10·9 ml.) covered with ether (52 ml.). After separation the aqueous layer was extracted with cooled ether, and the combined ethereal layers were washed three times with cooled water and promptly added to L-α-amino-*n*-butyrylglycine (1·75 g.) and $KHCO_3$ (4·36 g.) in water (22 ml.). The mixture was allowed to reach room temperature and stirred for 5 hr. The next day the aqueous layer was acidified, extracted with ethyl acetate (3 × 60 ml.), and the combined extracts were washed thrice with water and then extracted with 2% (w/v) Na_2CO_3 (7 × 10 ml.). The first three extracts were combined, acidified and extracted with ethyl acetate, and the ethyl acetate solution was washed with water, dried (Na_2SO_4) and concentrated to a volume of 15 ml. The *benzyloxycarbonyl tripeptide* (2·4 g.) crystallized, and was united with a further crop (0·3 g.) obtained from the last three fractions of the 2% Na_2CO_3 solution by repeating the fractionation, only the first two fractions obtained by extraction with 2 ml. of 2% (w/v) Na_2CO_3 being used. The product was purified by triturating with ethyl acetate (14 ml.); the residue (1·8 g.; 39%) had m.p. 166–168° (decomp.), not raised by recrystallization from a large volume of ethyl acetate, $[\alpha]_D^{20}$ – 19° (*c*, 3·2 in acetic acid); (Found: C, 53·4; H, 5·85; N, 9·5. $C_{19}H_{25}O_8N_3$ requires C, 53·8; H, 5·95; N, 9·9%).

γ-L-Glutamyl-L-α-amino-n-butyrylglycine (*ophthalmic acid*) (I). Benzyloxycarbonyl-γ-L-glutamyl-L-α-amino-*n*-butyrylglycine (1·7 g.) was suspended in acetic acid (5·8 ml.) and water (40 ml.) and hydrogenated in the presence of palladium black (0·7 g.) for 2·5 hr. The filtered solution was concentrated to a volume of 3 ml., seeded and slowly diluted with acetone (5 ml.); after a short time, the supernatant solution was decanted from the oil, and on standing *ophthalmic acid* separated as an oil which subsequently solidified and was dried *in vacuo* at room temperature (yield 0·8 g., 69%), m.p. 179–180° (decomp.), $[\alpha]_D^{20}$ – 29° (*c*, 2·4 in water); (Found: C, 44·7; H, 6·3; N, 14·5. $C_{11}H_{19}O_6N_3,\frac{1}{2}H_2O$ requires C, 44·3; H, 6·8; N, 14·1%). The water of crystallization was not lost at 100°.

A portion of the tripeptide was hydrolysed with 6N-HCl in a sealed tube at 107° for 16 hr. The hydrolysate was analysed by quantitative paper chromatography (Waley, 1957*b*), 72% (w/w) phenol–aq. 0·2% (w/v) NH_3 soln. being used as solvent; the molar ratios of the amino acids in the hydrolysate were: glycine, 0·98; α-amino-*n*-butyric acid, 0·93; glutamic acid, 1·0.

The tripeptide is readily soluble in water and sparingly soluble in most organic solvents; the partition coefficient (phenol–water) is 0·4. Ophthalmic acid forms a sparingly soluble copper salt from which it may be liberated with 8-hydroxyquinoline (Waley, 1957*b*).

Samples of natural and synthetic ophthalmic acid were submitted to Mrs D. M. Hodgkin, F.R.S., and Dr Beryl Oughton, who wrote as follows: 'Two series of powder photographs were taken, one of the free peptides, the other of the copper salts of the peptides.

'The powder photographs of the natural and synthetic free peptides were identical in line pattern and relative line intensity and clearly indicated the identity of the two preparations. Some difficulties were, however, encountered in the study of the copper salts. The first powder photographs taken of the natural and synthetic compounds were extremely similar and provided strong evidence of the virtual identity of the molecules present in the two crystals. They showed, however, small differences in the wide-angle distribution of a few powder lines. It is difficult to account for these; they suggest the existence of very slightly different crystal modifications, the occurrence of which may be favoured by impurities present or small changes in water content. A later specimen of the natural copper salt, although much discoloured and clearly grossly impure,

gave a pattern closely the same in line distribution as that of the synthetic specimen. This pattern showed much broadened lines, presumably owing to very small crystallite size.'

γ-L-Glutamyl-L-α-amino-n-butyric acid. Sodium nitrite (0·5 g.) in water (5 ml.) was added dropwise to a cooled, stirred solution of benzyloxycarbonyl-γ-L-glutamyl-hydrazide (1·5 g.) in water (12·5 ml.) containing 6N-HCl (5 ml.) covered with ether (25 ml.). After separation, the aqueous layer was extracted with cooled ether, and the combined ethereal layers were washed twice with cooled water and promptly added to L-α-amino-*n*-butyric acid (0·524 g.) and $KHCO_3$ (2 g.) in water (10 ml.). The mixture was stirred for several hours, and allowed to stand overnight. The acidified aqueous layer was extracted with ethyl acetate, and the combined extracts were washed with water and extracted with 2% (w/v) Na_2CO_3 (5 × 5 ml.). The first three extracts were combined, acidified and the oil was isolated by extraction with ethyl acetate. Hydrogenation was carried out in acetic acid (10 ml.) and water (5 ml.) in the presence of palladium black (0·15 g.) for 30 min. The filtered solution was concentrated, and the *dipeptide* precipitated by the addition of 3 vol. of acetone (yield 0·46 g., 38%). The analytical sample, recrystallized twice more from water–acetone, had m.p. 176–178° (decomp.); (Found: C, 45·9; H, 6·5; N, 12·3. $C_9H_{16}O_5N_2$ requires C, 46·5; H, 6·9; N, 12·1%).

In preliminary experiments the cheaper DL-α-amino-*n*-butyric acid was used; the product had the same chromatographic properties as γ-L-glutamyl-L-α-amino-*n*-butyric acid but, being a mixture of diastereoisomers, did not crystallize and was not characterized.

SUMMARY

1. The electrophoretic mobility of ophthalmic acid, a tripeptide isolated from calf lens, shows that the glutamic acid residue is γ-linked, and hence that ophthalmic acid is γ-glutamyl-α-amino-*n*-butyrylglycine.

2. This structure has been confirmed by the synthesis of ophthalmic acid.

3. The structure of opthalmic acid is compared with that of glutathione.

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